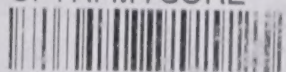


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Bacterial metabo.

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BACTERIAL METABOLISM

BACTERIAL METABOLISM

BY

MARJORY STEPHENSON, Sc.D., F.R.S.

READER IN CHEMICAL MICROBIOLOGY IN THE UNIVERSITY
OF CAMBRIDGE

WITH DIAGRAMS

THIRD EDITION



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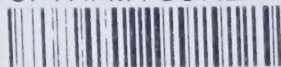
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Bacterial metabo.

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PREFACE TO THIRD EDITION

SINCE 1939 the progress of bacterial metabolism has been startlingly rapid. This is due partly to the greater number of workers in the field, partly to the introduction of new techniques, but most of all to the evolution of new concepts involving fresh experimental approaches.

One of the most important technical innovations has been the use of isotopes ; so far the subjects most favourably affected have been fermentation, oxido-reductions involving CO_2 , and nitrogen fixation. By the use of labelled carbon it has been possible to clear up many obscure patches in the intermediary processes of bacterial fermentations which could not otherwise have been settled with certainty. The same technique has led to the recognition that CO_2 is one of the principal oxidising agents of anaerobic life ; its reduction to methane and to acetic acid has now been established together with the fact that such reductions supply not only the energy but also the carbon compounds for cell synthesis. In the case of nitrogen fixation the use of N^{15} has enabled the course of nitrogen both fixed and free to be followed within the cell though the mechanism of fixation itself is still obscure.

A subject in which gratifying progress has been achieved is that of filterable toxins. Nine years ago the nature and mode of action of these substances was enveloped in a mist comparable to that which in the twenties obscured investigation into the nature of enzymes. Now some eight or ten toxins have been partially or completely purified and the mode of action of some clearly disclosed.

The advances so far mentioned have been along lines laid down long ago but during the last few years a fresh view of bacterial metabolism has been opened up. Information is now being rapidly gained on the course of the biochemical processes leading to cell synthesis ; such studies are peculiar to microbiology though certainly of wider application ; they owe their success to the use of biological material which is prone to biochemical variation and

tolerant of interference with its normal biochemical habit. This new stream of knowledge has its origin in several sources : microbial genetics, nucleic acid metabolism, adaptive enzyme formation, function of growth factors, the intracellular changes resulting from chemotherapeutic agents, antibiotics and other cell poisons, and interference with metabolism resulting from the introduction into the cell of chemical analogues of essential cell metabolites. All these are contributing to produce a picture—at present incomplete and patchy—of the biochemical machinery of growth. We seem, in fact, to be witnessing a transition from katabolic to anabolic studies, made possible only by the use of the microbe as experimental material.

These studies are only beginning but are proceeding so rapidly that the period during which this edition has been passing through the press¹ has sufficed to make it a back number so far as some of these aspects are concerned.

In preparing this edition, I have, in spite of caustic criticism, followed my original policy with respect to nomenclature, viz. that of using the name of the organism employed by the worker from whom I quote. I deplore the irrational appearance this gives to the book but I can think of no other course which will not involve me in attempting to re-identify all the organisms discussed, a task which, in the case of early work, is quite beyond me.

I have many friends whose help I must acknowledge in addition to those who have aided me in preparing previous editions ; these are :

In respiration and fermentation T. Mann and D. M. Needham ; in polysaccharides D. J. Bell and Professor M. Stacey ; in filterable proteases and toxins W. E. van Heyningen ; in nitrogen metabolism E. F. Gale ; in photosynthesis S. R. Elsdén ; in nucleic acid Dr. C.-E. Hedén, who permitted me to read unpublished work (Malmgren and Hedén, 1947) and to reproduce Figs. 3 and 4 in Chapter VI. In the subject of bacterial nutrition I have profited greatly by Dr. Knight's review frequently quoted and by a preview of his forthcoming monograph on the same subject (Acad. Press Inc., New York).

My thanks are also due to Mrs. W. Cohen for secretarial aid.

¹ The script of this edition left my hands in February 1947.

Finally for the expert and laborious work of preparing the index I have for the third time to thank my earliest colleague Margaret Anderson.

To serve as landmarks in the progress of thought on microbiology, I am reprinting the prefaces to the editions of 1930 and 1939.

Bacterial metabolism is now such a wide study that it is no longer convenient for one person to attempt to cope with all its branches ; I can confidently assert that this is the last edition which will appear over the name of one author.

M. S.

CAMBRIDGE

March 1948

PREFACE TO SECOND EDITION

THE rapid advance in our knowledge of bacterial biochemistry since the appearance of this book in 1930 has made the preparation of the new edition as formidable a task as that of writing the first. It has also rendered *Bacterial Metabolism* unsuitable for publication as a monograph, the exhaustive treatment of the whole subject by one author being now impossible. The Publishers have therefore agreed to remove the book from among the "Monographs on Biochemistry" and to re-issue it in an enlarged form as an advanced textbook.

In these circumstances it has become of interest to note some of the changes in outlook which have occurred during the past eight years. While substantial advances have been made along the entire front, movement in certain sectors has been extremely rapid, in some cases taking a direction quite unforeseen in 1930.

In the problem of bacterial growth advances have been made along new lines. Happily this subject now attracts mathematicians and statisticians less than formerly but has passed into the hands of biochemists interested in problems of nutrition; this has led to results of both theoretical and practical importance and has revealed *inter alia* that the complex and peculiar media employed by bacteriologists in the cultivation of "difficult" pathogens are rendered necessary owing to the inability of many parasitic organisms to synthesise for themselves certain molecules essential for growth. Some of these substances are identical or closely related to certain vitamins known in the animal whilst others appear for the first time as substances of biological importance. Such discoveries are double-edged; the fact that co-enzyme I or II must be supplied ready-made to organisms of the *influenza* group whilst other organisms synthesise it for themselves, supplies a means of studying its rôle in metabolism; nutritional and metabolic studies thus supplement each other. The study of vitamins in the animal has had a long start, but the rapid accumulation of knowledge concerning accessory food factors for bacteria has already disclosed the necessity for substances not yet known to play a part in animal metabolism. It is not unlikely that in the future knowledge gained in this field may help to solve problems in animal nutrition just as hitherto the reverse has been the case.

In the field of bacterial fermentations, facts have accumulated fast largely owing to the stimulus gained from the great advances

made in our knowledge of alcoholic and muscle fermentation ; the main check to advance has been the difficulty of making active cell-free extracts of bacteria comparable to those obtainable from certain strains of yeast.

The study of bacterial enzymes shows that though they conform to the same laws as do those belonging to the animal and vegetable world their range of action is incomparably wider. Numerous systems completely missing in animals and plants flourish in different groups of bacteria, as, for example, the enzymes concerned with the utilisation of molecular hydrogen and with the reduction and oxidation of many inorganic molecules. It would not be unsafe to predict that all the enzymes found in the animal will ultimately turn up in some bacterium or other whilst those found only in bacteria will be far more numerous.

In 1930 the problem of nitrogen fixation had for some years remained almost stationary, but during the last few years it has been fiercely attacked from several quarters ; so great have been the advances made that there is every hope that in a short time all the intermediate stages in this remarkable process will be unravelled.

Finally it is a satisfaction to record that the present decade has witnessed an event in bacterial chemistry of first-class biological importance, namely, the discovery of bacterial photosynthesis. The demonstration of a wide and varied group of bacteria relying on solar radiation as a source of energy yet employing for the reduction of carbon dioxide compounds quite distinct from the one in use by the green plant, amounts in fact to the revelation of a new mode of life, and ranks with Winogradsky's discovery of chemosynthetic organisms at the end of the last century. This is yet one more example of the rich variety of biochemical mechanisms employed by bacteria.

In the matter of bacterial nomenclature I have kept to my original plan when quoting work of using the names employed by the original author ; this sometimes leads to an organism appearing under two slightly different appellations in adjacent passages, an apparent absurdity which is unavoidable.

The prefixes *d*- and *l*- are used throughout to denote the configurational series, + and - the rotation.

In rewriting this book I have had generous help from many quarters ; in particular I must thank the following : Drs. Malcolm Dixon, D. E. Green and T. Mann for help in the chapter on "Respiration" ; Drs. D. M. Needham, D. J. Bell and Mrs. C. Mann in the chapter on "Carbohydrate Breakdown" ; Dr. D. D. Woods in the section on "Amino-acids." The chapter on "Nutrition" owes much to Dr. B. C. J. G. Knight for criti-

cism and advice. Dr. Dean Burk kindly read the script of the chapter on "Nitrogen Fixation," and Professor A. I. Virtanen generously allowed me access to material, then unpublished, used in his lectures delivered at the London School of Hygiene and Tropical Medicine in November 1937.¹

I am grateful to Dr. D. D. Woods for sharing in the task of proof correcting, and to Mrs. Pauline Baldwin for efficient secretarial aid; I have again to thank my friend and former collaborator, Mrs. Anderson, for making the index. I am indebted to Mr. H. Mowl for preparing many drawings. To many other colleagues who have helped me I here return my thanks.

I have now a wider debt to acknowledge. Bacterial biochemistry has been made possible in Cambridge by financial support from the Medical Research Council now extended over a number of years. We have been singularly fortunate in being grafted into the School of Biochemistry and so enabled to profit by contact with workers investigating many branches related in varying degree to our own studies. As biochemistry advances it unfolds a bewildering complexity yet at the same time displays a frequent repetition of pattern; only by co-operative working and thinking can progress be achieved. It has been the peculiar happiness of this department to work with a Professor who shares his own unique inspiration with his colleagues, and fosters in them the same spirit of co-operation.

M. S.

THE BIOCHEMICAL LABORATORY

CAMBRIDGE, *September 22nd*, 1938

¹ A. I. Virtanen, *Cattle Fodder and Human Nutrition*. Cambridge University Press, 1938.

PREFACE TO FIRST EDITION

THE aim of this book has been to choose from the mass of data on the chemical activities of bacteria facts which may help us to gain an insight into the essential chemical processes accompanying the life of the organisms concerned. To form any coherent picture of these happenings is at present beyond our powers; we are indeed in much the same position as an observer trying to gain an idea of the life of a household by a careful scrutiny of the persons and material arriving at or leaving the house; we keep accurate record of the foods and commodities left at the door, and patiently examine the contents of the dustbin and endeavour to deduce from such data the events occurring within the closed doors. Much of our time must of necessity be spent on the study of irrelevancies, and in the sorting out of the important from the insignificant many mistakes must be made.

In the selection of material for consideration in this book doubtless much has been omitted which will turn out to be of importance and much included which will prove to be irrelevant; but it is time that an attempt should be made to arrange the scattered data in order to appraise our knowledge of bacteria as living organisms apart from their rôle as disease germs or the bearers of commercially important catalysts.

In the attempt to gain some idea of bacterial physiology, reference to related problems amongst other orders of living systems is necessary; only thus can the material be viewed in perspective; for this reason reference to facts and theories gained in other fields of biochemistry has occasionally been made, and for this no apology is tendered.

The place of bacteria in evolution is a question very difficult of approach; we have, for example, no idea whether the forms familiar to us resemble primitive bacterial types or whether, like modern animals and plants, they are the successful competitors of the ages. Perhaps bacteria may tentatively be regarded as biochemical experimenters; owing to their relatively small size and rapid growth variations must arise very much more frequently than in more differentiated forms of life, and they can in addition afford to occupy more precarious positions in natural economy than larger organisms with more exacting requirements. No large animal or plant, for example, could hope to survive if obliged to depend solely on the oxidation of ammonia or sulphur for its

energy. The profitable sources of energy have been seized upon and probably fully exploited by the green plant and the animal respectively; the autotrophic bacteria lead a hard and precarious existence due to the adoption of a type of metabolism ill-adapted to life on this planet, and only possible to organisms whose demands are small. Apart from their importance in soil economy, however, the autotrophic bacteria are of intense interest as suggesting courses which physiological evolution might have taken had a slightly different equilibrium established itself in the inorganic world.

Heterotrophic bacteria which, in the main, resemble the animal in their chemical mode of life, yet display many chemical powers never met with among more differentiated forms of life; the question whether these powers are all biologically significant, that is inevitable accompaniments of biological processes, or whether some of them are merely the result of enzymic activity unrelated to physiological events, is at present matter for speculation.

With regard to nomenclature it has not been found practicable to adopt throughout the system recommended by the American Society of Bacteriologists, since it is difficult to be certain how to designate by their modern titles many of the species mentioned in the early work quoted; for this reason the names have been allowed to stand in the form in which they occur in the literature cited.

Among earlier publications I am particularly indebted to *Allgemeine Mikrobiologie* of Kruse (1910) and also to *Die Einwirkung von Mikroorganismen auf die Eiweisskörper*, by Paul Hirsch (1918).

In conclusion, it is a pleasure to thank my colleagues past and present for the help and stimulus I have received from numerous discussions and free interchange of ideas on matters biochemical. I have also to thank in particular Dr. Malcolm Dixon for invaluable advice and help in the section of Chapter III dealing with the subject of oxidation and reduction potential.

From my friend and former collaborator, Margaret Whetham (now Mrs. Anderson), I have received valuable criticism throughout the book, and I have also to thank her for help in correcting the proof sheets.

Finally, and especially, my thanks are due to Professor Sir Frederick Hopkins, at whose suggestion the book was written and to whose influence alone I owe the incentive to think on biochemical matters.

THE BIOCHEMICAL LABORATORY
CAMBRIDGE, March 8th, 1929

M. S.

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“Ruder heads stand amazed at those prodigious pieces of nature, whales, elephants, dromedaries, and camels ; these, I confess, are the colossus and majestick pieces of her hand ; but in these narrow engines there is more curious mathematicks ; and the civility of these little citizens more neatly sets forth the wisdom of their Maker.”—SIR THOMAS BROWNE, 1642.

CHAPTER I

INTRODUCTION

FOR the introductory chapter to the third edition of this book the material bears no comparison with what was available in 1929. Since that date biochemistry has achieved so many notable advances applicable to the understanding of cell metabolism that the field has become extended and enriched beyond recognition. A fair measure of agreement might now be found for the definition that bacterial metabolism is the interpretation of the physiological life of the bacterial cell in terms of biochemistry and biophysics both in respect of its own growth and reproduction and also of its action on its chemical and biological environment.

The position now seems to demand a glance back at the past and a retracing of the path by which we have reached our present position, an effort to appraise the present and even perhaps an attempt to peer into the future.

The Pasteur period

In November 1946 a memorable congress assembled in Paris to commemorate the fiftieth anniversary of the death of Pasteur. At this meeting, attended by delegates from every civilised country, the present position of all branches of microbiology was appraised, the contributing members having been invited to point out in what way developments in their special fields owed their initial impetus and inspiration to the work of the Master. The results must have startled the least imaginative; every branch of microbiology, whether pure or applied, was in turn shown to have owed its origin to some phase of the work of Pasteur or of his immediate pupils. It is therefore inevitable that a sketch of the initiation and progress of bacterial metabolism should start with the consideration of some aspects of the work of the man who laid its foundation.

Seldom has a science developed with such suddenness as microbiology or owed its beginning so exclusively to the work of one man. This suggests that by 1857, the year in which appeared Pasteur's first study on fermentation,¹ the birth of microbiology was somewhat overdue. Before considering this hazy suggestion it is necessary to examine briefly the views of Pasteur's immediate

¹ Pasteur, 1857.

predecessors in the study of fermentation and the layout of scientific opinion at this period.

Yeast fermentation was of course known and practised in prehistoric times ; this surprises no one who has lived and prepared food in tropical or subtropical countries, where every sandstorm is followed by a spate of fermentation in the cooking-pot. Every savage tribe—save perhaps the Esquimaux—has to-day its characteristic fermented food prepared according to a traditional method. Such knowledge of microbial activity preceded by thousands of years the recognition of the existence of microbes. The latter was first brought to light in the seventeenth century by the work of the Dutch microscopist, Antony van Leeuwenhoek, who succeeded in grinding lenses of sufficient magnifying power to reveal bacteria. He made accurate observations of bacilli, cocci and spirochaetes from the mouth, recorded motility, noticed proliferation and made approximate measurements, using sand-grains as a standard of comparison. These leave no doubt that it was indeed bacteria that he observed.¹ This discovery, however, did not lead immediately to the birth of microbiology as a science, for reasons we shall shortly consider, but lay dormant till late in the nineteenth century.

The first work leading up directly to that of Pasteur was the chemical work of Gay Lussac² on the quantitative nature of the change of sugar in fermentation and the establishment of the equation which bears his name :



About 1837 the biologists entered the field and it became recognised through the work of Cagniard-Latour,³ Schwann⁴ and Kützing⁵ that yeast was a living organism responsible for the chemical change known as fermentation. These men were, however, observers rather than experimenters and were opposed heavily by the chemists of that time—Berzelius, Wohler and Liebig—and it is to be remembered that the period was one in which chemistry was in the ascendant. Berzelius opposed the biological concepts with the theory that fermentation was a phenomenon of contact catalysis due to a non-living catalyst, whilst Liebig believed that it was dependent on the decay of dead animal or vegetable material. By 1857 the three biological workers were dead or old and the chemists held the field. In 1857 came the publication of Pasteur's first paper, *Mémoire de la Fermentation dite lactique*,⁶ and it is interesting to inquire exactly what led the Professor of Chemistry in the University of Lille to turn from his

¹ Dobell, 1932.

⁴ Schwann, 1837.

² Gay Lussac, 1810.

⁵ Kützing, 1837.

³ Cagniard-Latour, 1837.

⁶ Pasteur, 1857.

successful researches in crystallography to the study of fermentation.

It appears that in 1855 the course of Pasteur's investigations led him to strike a snag. From the crude fusel oil derived from fermentation he obtained two amyl alcohols of the formula $C_5H_{11}OH$, the one levorotatory and the other optically inactive. The optically inactive alcohol resisted all efforts to resolve it into two components and therefore presented the first exception to the rule that where an optically active and an optically inactive form of the same compound exist the latter can be resolved into two optically active forms.¹ Pasteur tells us that it was in the hope of solving this problem that he turned his attention to the study of fermentation and there is no record of his ever returning to crystallography; nor in fact did he solve the problem which lured him into the field. This was achieved by Felix Ehrlich² in 1907 when he showed that the inactive alcohol is obtained by the action of yeast on *l*-(-)-leucine and the *l*-rotatory alcohol in a similar way from *l*-(+)-*iso*-leucine, the latter alcohol alone possessing an asymmetric carbon atom.

As a result of his first studies on fermentation Pasteur published in 1857 the paper already alluded to, which marks the birth of the science of microbiology. In this famous paper he made the following points which cut clean across the views current among the chemists of his day:

1. That the lactic fermentation was due to a living cell or ferment (the words cell and ferment were used interchangeably; the cell in Pasteur's view *was* the ferment). This constituted a challenge to Berzelius.

2. That decay either of dead cells or added nitrogenous matter had nothing to do with fermentation but served merely as food for the growing cell. This was a challenge to Liebig.

3. During fermentation a multiplication of cells occurred distinct in type from those causing alcoholic fermentation. This amounted to the discovery that distinct microbial species bring about different chemical reactions.

4. That if during fermentation the cells were skimmed off and added to fresh sugar solution a very rapid fermentation occurred, especially if the acid formed were neutralised by chalk. This was the first use of resting cell suspensions.

5. In addition to lactic acid, butyric and succinic acids were formed.

6. Natural fermentations were liable to be mixed, i.e. in part lactic and in part alcoholic. This could be controlled by attending

¹ Pasteur, 1855.

² Ehrlich, 1907²(2).

to reaction, an acid reaction favouring alcoholic and a neutral reaction lactic fermentation.

7. Yeast multiplication could be completely inhibited by onion juice. This was probably the first recorded observation of an antibiotic.

Pasteur's comments on his own papers are revealing :

If someone tells me that in making these conclusions I have gone beyond the facts I reply : " This is true, that I have freely put myself among ideas which cannot be rigorously proved. That is my way of looking at things. Every time a chemist concerns himself with these mysterious phenomena and every time he has the luck to make an important step forward he will be led instinctively to attribute their prime cause to a class of reactions in harmony with the general results of his own researches. That is the logical course of the human mind, in all controversial matters. Furthermore it seems to me from the standpoint which I have reached in my knowledge of the subject that whoever will impartially judge the results of this work . . . will agree with me in recognising that fermentation is associated with the life and structural integrity of the cells and not with their death and decay ; neither is it a contact phenomenon in which the change in the sugar takes place in the presence of a ferment without the latter giving or gaining anything."

No passage in scientific literature is of more philosophical significance. Pasteur had worked on fermentation for two years and then put forward views which he admits were not satisfactorily proved, and were directed and coloured by the tendencies of his own mind. " All scientists," he says in effect, " work like this." If *we* look into our own minds we shall probably be led to admit that this is true ; we select among many possible explanations of new phenomena those which fit in best with ideas already familiar to our minds. If we are biochemists we seek for explanations of chemotherapeutic phenomena in terms of enzyme chemistry, whereas others may look for them among surface phenomena.

But what an admission ! No wonder there broke over Europe a first-class scientific row.

But what exactly did Pasteur reject and what select to suit the natural disposition of his mind and the results of his experiments ? First he rejected the idea of Liebig that fermentation occurs as a result of contact with decaying matter. As this idea is in fact untrue it died as rapidly as its association with the great name of Liebig would permit. There remained the view that fermentation occurs as a result of contact catalysis. In 1946 we are in a position to know that this explanation is in close accord with facts and it is interesting to see why Pasteur rejected it. He ignored it because it *meant nothing to him*. Contact catalysis in this con-

nction suggests to us the chain of enzyme-catalysed reactions by which we now know fermentation is brought about. But in Pasteur's day enzyme action in this sense was unknown and was restricted to simple hydrolytic processes such as the action of diastase or invertase and other hydrolytic enzymes secreted by cells or tissues and acting when separated from the cells which produced them.

So Pasteur adopted what may be called the physiological point of view. He regarded the yeast cell or the lactic bacterium as a living organism with a life to lead and an axe to grind. Fermentation he believed was a process *necessary* to the life and multiplication of the cell. He reiterated times without number that fermentation is necessarily related to the life of the cell and as a corollary, does not occur in the absence of the living cell. The choice of this viewpoint was clearly and admittedly instinctive but it was accepted, and throughout Pasteur's life the whole of microbiology was interpreted in its light. Every fresh discovery of the chemical effects of bacteria was interpreted in terms of cell physiology. *Thus was bacterial metabolism born.* Now supposing Pasteur had chosen the contact catalysis view, where would it have led? The oxidation of NH_3 would have been regarded as a contact phenomenon brought about by a certain type of cell and left at that. This view would never have led to the discovery of a class of living organisms utilising the energy derived from this process for the fixation of CO_2 ; the autotrophs would not have been discovered by that means. Nitrogen fixation would have been discovered but regarded as an accidental property of certain cells, and its relation to the protein supply of the organism would hardly have become apparent; we can multiply these instances. No one would have interpreted fermentation in terms of *la vie sans air*; the study of anaerobiosis would have disturbed no one.

So Pasteur was pragmatically right. In effect he made his choice because physiology and organic chemistry were adequate for his purpose and biochemistry was too infantile and undeveloped. The weakness of Pasteur's position lay in the fact that he made no attempt to explain the mechanism by which the cell brought about those acts, oxidation, fermentation, etc., which he maintained were essential to its life. He seems, moreover, to have been unaware that such an explanation was called for, possibly because he instinctively realised that he was in no position to provide one.

But if Pasteur was quite happy in this position some of his contemporaries were not. Moritz Traube (1877),¹ for example, produced a theory of fermentation, and indeed of the chemical

¹ Traube, 1877.

activity of all cells, remarkable for its inclusion of the knowledge then available and for its forecast of later discoveries. According to him the cell causes fermentation and other chemical events by means of substances of a protein character with a definite chemical composition. These substances, themselves remaining unchanged, were, according to Traube, responsible for the vital chemical changes of higher and lower animals. Traube included all the known chemical activities of microbes in his purview; the oxidation of alcohol and ammonia, he said, is carried out first by the transfer of O_2 to the ferment and then to the oxidisable substance. He pictured fermentation as the transfer of oxygen from one part of the sugar molecule to another, culminating in the highly oxidised CO_2 and the highly reduced alcohol. Had Traube chosen the transfer of hydrogen instead of oxygen his explanation would have sufficed till about 1932; it is in fact a remarkably correct picture of enzyme action formulated by guesswork.

But Pasteur barely attended to this paper; it simply didn't interest him. This was, I believe, because it was purely speculative and not founded on experimentally acquired knowledge. The enzyme chemistry of that period was mainly confined to extra-cellular hydrolytic enzymes such as amylase, maltase, invertase and some crude digestive juices, and was not equipped to cope with cellular oxidations, fermentations and putrefactions. Hence Traube's contribution modified contemporary microbiology but little—it was forty or fifty years before its time; when that time had elapsed Traube's views were forgotten. It is always thus with guesses unsupported by experimental evidence. There is here an excuse for considering what are the factors which at any given time determine the direction of scientific advance. I am of course aware of the so-called materialistic view that the directive stimulus is derived from the economic requirements of the community when these are recognised as such by the dominant section of the people. For me this view has been reiterated *ad nauseam* and I do not believe that it is the primary controlling force. For instance, there has never been a time during the historical period when the control of recurrent famines would not have paid the community who solved the necessary problems; the same is true for the control of pestilences. Yet research into these problems did not attract the great seventeenth-century investigators who concentrated rather on physics. The reason, of course, is obvious, viz. that every line of investigation must await the development of knowledge in certain related fields and, until this has reached a certain level, it cannot be successfully attempted. Agriculture awaited the development of botany and chemistry before it could solve problems of production. Epidemiology waited on

bacteriology which in its turn depended on physiology and chemistry.

Pasteur could not have carried out his work till physiology had attained clear conceptions about respiration, absorption, energy requirements and the like ; and physiology in its turn depended on the elementary concepts of chemistry and physics before it could apply these to elucidating the machinery of the animal body. All this sounds too trite to deserve attention, nevertheless in the course of the history of our subject we get occasional instances of discoveries made in advance of their time and it is interesting to see what then happens. Take, for example, the discovery of microbes by Leeuwenhoek already mentioned. What did he and his contemporaries do with this discovery ? Just nothing. In spite of its wide publicity and the interest it evoked when reported to the Royal Society of London it led to no further advance because science was not sufficiently developed to make use of the observation. In the absence of basic knowledge about chemistry it could not be related to fermentation or putrefaction ; without physiology it could not be related to cell life or contagious disease. Leeuwenhoek may have been the father of microscopy, but he was in no sense the father of microbiology.

To take another example nearer our own day—the discovery of cytochrome by MacMunn in 1886.¹ MacMunn believed this substance to be an important oxygen-carrying mechanism but he had no conception of enzyme action and lacked the means to study the cytochromes from that point of view. Hence, though he convinced himself of the importance of his discovery, he lacked the technique to take the study to the point where it could carry conviction to others. This waited for thirty-eight years when, in 1925, enzyme chemistry was sufficiently advanced for the task, and Keilin was able to interpret the work in the light of his own masterly researches.²

Scientific discoveries made in advance of their time are like long salients in an army by which a position may be captured ; if, however, the main army is too far behind to bring up the necessary supplies the successful advance is cut off and may be forgotten and the position has then to be recaptured at a later date. This explains why Pasteur's physiological approach to problems of microbiology was so successful and why Traube's explanation in terms of enzyme chemistry—equally true and much more explicit—failed.

In 1895 Pasteur died ; two years later the Buchners³ prepared a cell-free juice which fermented sugar. Here then was fermentation without a living cell ; the weak place in Pasteur's theory had

¹ MacMunn, 1886.

² Keilin, 1925.

³ Buchner, 1897.

betrayed it and the physiological conception of fermentation temporarily collapsed.

Owing to the prestige of Pasteur and the wide acceptance of his tenets, the Buchners' discovery created a sensation out of all proportion to its importance. Actually in the conceptions of those days it could have been readily explained as a mechanism surviving the destruction of the cell which had built it; compared with the cells from which it was derived the juice was weak and its activity short-lived. Scientifically it led directly to few important results, of which probably the work of Harden and Young¹ was the most important; but its inhibitive effect was surprising, for subsequent to its publication attempts to interpret chemical occurrences in terms of cellular physiology fell into abeyance as far as bacteriology was concerned for roughly twenty years.

During the Pasteur period the role of organic chemistry in the development of microbiology presents many points of contrast to that of biochemistry. It was highly developed when microbiology started, and was from the first adequate to meet the demands of the new science. Methods for the isolation, identification and estimation of fermentation products were available as soon as they were wanted, and in the hands of such men as the Salkowskis, F. Ehrlich, Brasch and Ackermann were successfully applied to many newly found products of putrefaction.

Medical advances

Meanwhile medicine was entering into its inheritance of Pasteur's work. Perhaps the most characteristic figures in this advance were Koch and Ehrlich. Koch's great achievements in laying the foundation of practical medical bacteriology were greatly aided by his simplicity of outlook; his famous postulates are strictly utilitarian and aimed at and succeeded in securing the correct attribution of the agent to the disease, thus avoiding serious mistakes in medical practice. No guesses as to the "how" are hazarded by this great man; he possessed the empirical outlook and aimed at the perfect technique. He was the right man at the right time and medicine probably owes as much to his limitations as to his great gifts. Ehrlich was very different; his immunological and serological observations cried aloud for chemical interpretation but neither the biochemistry nor the physical chemistry of the day was equal to the task. Ehrlich's principal need was the protein chemistry developed between 1930 and 1940; immunochemistry now draws on crystallography, electrophoresis, ultracentrifugalisation and the ultramicroscope for the interpretation of its data. Since all this was lacking in Ehrlich's day two

¹ Harden & Young, 1905.

alternatives were open to him: to proceed empirically as did Koch, or to construct an artificial chemistry to fit his observations. He adopted the latter course. Many of Ehrlich's concepts and most of his terminology are now obsolete, but they served as scaffolding for his valuable experimental work and it would have been impossible for a mind of his type, working on immunological data, to have proceeded without something of the kind. With later developments of protein chemistry this scaffolding has been replaced by actual knowledge regarding the true nature of the antigen-antibody reaction permitting the rapid advances that we are now witnessing.

The intermediate period and the rise of enzyme chemistry

About 1920 the study of enzyme action began to gather momentum. It was no longer confined to the soluble hydrolytic enzymes but extended to the various phases of intracellular oxidations and reductions. Owing to the difficulty of separating the enzymes concerned from the cells the early studies were mainly on enzyme kinetics and the preparations used were concentrates prepared from animal or plant tissues.

This led to similar studies in bacterial chemistry by the use of washed bacterial suspensions—the "resting bacteria" of the Cambridge School—in which intact but non-proliferating bacteria were treated as tissues or tissue extracts. A somewhat similar use of yeast enabled the study of fermentation to be resumed. The views of Pasteur had by now been modified and extended; fermentation was recognised as the function of a living, but not necessarily of a multiplying, cell. It was further realised that the process was not a single reaction but a chain of reactions each probably controlled by a single endocellular enzyme. Since such enzymes were still inseparable from the cell their individual action was made apparent largely by the use of cell poisons and enzyme inhibitors. In so far as it concerned yeast Neuberg was one of the principal exponents of this line of work, from which he evolved a comprehensive scheme purporting to explain the course of alcoholic as well as of many bacterial fermentations. This scheme influenced work for a surprisingly long period—till 1932—and stimulated work on bacterial fermentations, particularly in the Delft School. Neuberg's scheme was eventually shown to be largely erroneous and was superseded by that due jointly to Embden, Parnas and Meyerhof, in which the chain of reactions was shown to occur in phosphorylated molecules.

During the same period the use of bacterial suspensions had been extended to the study of a large number of enzyme processes in addition to oxidations and reductions, but the chemical nature

of enzymes was still unknown, and though kinetics were studied and products isolated the intimate nature of enzymes and their action were obscure.

Moreover these studies had so far little bearing on bacteria as agents causing disease ; their merit lay in initiating the study of the mechanisms of microbial life. The impact of organic chemistry during this period occurred in several places. Chief among these was on immunology and came to supplement the discovery of the importance of bacterial polysaccharides as the bodies conferring specificity on bacterial antigens. Along with this discovery came the demand for knowledge of the structure of the newly isolated polysaccharides, starting with the identification of the pneumococcal (Type III) polysaccharide as 4- β -glucuronosidoglucose. Carbohydrate chemists have successfully collaborated with bacteriologists in elucidating the structure of these important substances.

The period 1930-1946

The work of this period is not yet history and most of the subsequent chapters in this book are concerned with discussing it ; an attempt to summarise it here is therefore out of place. The lines of work characteristic of the previous period proceeded with gathering momentum, but the front has been extended largely owing to fresh biochemical developments. One of the chief of these has been the growth of protein chemistry due to contributions from organic and physical chemists and crystallographers. A deeper knowledge of protein chemistry was bound to have enormous repercussions on cellular biochemistry owing to the superimportance of these compounds in cell life. As early as 1926¹ it was announced that the enzyme urease had been isolated and crystallised and that it was a protein. Thus ended the doctrine that enzymes were of such instability and of such a fugitive character that their chemical study was doomed to failure. By the end of 1937 ten enzymes had been isolated in the form of crystalline proteins. This period has witnessed also a greatly extended knowledge of fermentation, bacterial nutrition, photosynthesis, chemotherapy, viruses and bacterial toxins, a discussion of which would merely serve to anticipate their more detailed treatment contained in subsequent chapters.

But in addition something new is appearing from several directions and this concerns, not the mechanism by which the cell breaks down its chemical environment and so obtains energy, but some insight into the processes of growth. Knowledge is developing concerning the mechanism by which the nucleus or the nuclear

¹ Sumner, 1926.

material controls the biochemical construction of the growing cell, of how the antigen deflects the course of the synthesis of globulin so as to give rise to the antibody. The related problem of the mechanism at work in the production of adaptive enzymes is showing signs of cracking; the study of the action of chemotherapeutic agents and antibiotics is contributing unexpected information on intracellular synthetic mechanisms. These and other signs and portents announce that the attack on the biochemical mechanism of growth and inheritance has begun along several lines; this could not have been said ten years ago.

Allowing for the increasing momentum of the advance, the number of interested workers in the field and the recent advances in techniques available, it seems reasonable to hope that within a comparatively short time—say twenty-five to fifty years—a biochemical description of cell growth may be laid out before us.

With the attainment of such knowledge will the story then have lost its interest? For some doubtless this will be true; when a tract of country has been adequately surveyed it loses interest for the explorer and becomes the property of the common man. It is for many of us incomparably more interesting to be wandering in a half-light uncovering hidden truths, bumping into others similarly occupied and adding to a body of knowledge to which one's friends and contemporaries are contributing, than to be reading a complete account of established facts. Human anatomy must have been vastly more interesting to Vesalius and Leonardo da Vinci than to the student of to-day and the inheritance of unit characters and the phenomenon of dominance doubtless excited Gregor Mendel more than the modern student of genetics. Few people unite a love of scientific investigation with a vivid historical appreciation.

We must therefore face the fact that biochemistry and microbiology, as we know and envisage them, will in the course of time become cold stars and that with the modern rate of progress the time this will take will probably be measured in decades rather than in centuries. By then the inquiring mind of man will have moved into fresh fields whose character we can only dimly guess at. Meanwhile we are entering the most interesting phase of biochemistry and microbiology and students of the immediate future may anticipate a rich reward for their labours.

CHAPTER II

RESPIRATION

AEROBIC OXIDATION

LIKE many scientific terms the word "respiration" has shifted its meaning concurrently with increase in knowledge. Originally coined for mammalian physiology to denote the passage of air into and out of the lungs, it later became extended to cover the transference of O_2 to and CO_2 away from the tissues. As the centre of interest shifted from the phenomena of gaseous exchange to the mechanisms by which cell constituents become oxidised, the term again extended its meaning to embrace the more detailed and deeper biochemical studies included in the term "tissue respiration." Here the term extended in scope also to include plants and those micro-organisms for which molecular oxygen is required to carry out life processes. Furthermore, as it became clear that the chief significance of biological oxidation lies in the energy liberated for the use of the growing and functioning cell, the term "respiration" was once more extended to cover exergonic reactions not involving O_2 and the term "anaerobic respiration" came into use. It is only in its wider extension that the term "respiration" can be used of micro-organisms and for practical purposes it can be used to denote any chemical reaction, aerobic or anaerobic, by which energy is liberated by the cell.

Thermodynamical terms employed

It is well first to draw attention to the fact that the heat of combustion or heat of reaction does not necessarily represent the energy available to the cell for synthetic purposes, and it is necessary to introduce the conception of free, as distinct from the total, energy of a reaction. For this purpose the terms used by Lewis and Randall¹ will be employed as follows:

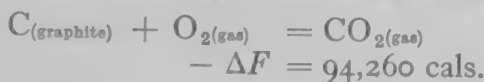
- I. $-\Delta H$ = Heat of reaction or total heat evolved in a reaction carried out at constant temperature and pressure.
- II. $-\Delta F$ = Free energy, i.e. the maximum amount of work which can be obtained from I in the same conditions of temperature and pressure.

¹ Lewis & Randall, *Thermodynamics*, New York, 1923.

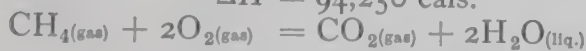
III. $-T\Delta S$ = The energy not convertible into work in the given conditions, where $-\Delta S$ is the decrease in entropy.

Hence $-\Delta H = -\Delta F - T\Delta S$.

In many cases of decompositions effected by bacteria, $T\Delta S$ is relatively small, that is ΔH approximates to ΔF , and in such cases little serious error arises from using the heat of reaction as equivalent to $-\Delta F$; but in certain cases the two values differ widely, instances even occurring when a negative heat of reaction is accompanied by an increase in free energy. As illustrations the following examples may be quoted, the conditions being a pressure of 1 atmosphere and a temperature of 25°C . ($298^\circ \text{Absolute}$):

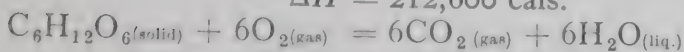


$$-\Delta H = 94,250 \text{ cal.}$$



$$-\Delta F = 194,600 \text{ cal.}$$

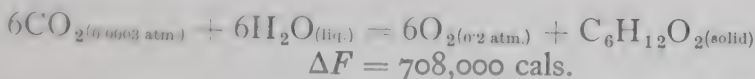
$$-\Delta H = 212,600 \text{ cal.}$$



$$-\Delta F = 685,800 \text{ cal.}$$

$$-\Delta H = 674,000 \text{ cal.}$$

The pressure under which the reacting gases work materially affects the amount of the free energy change, though not materially the ΔH ; thus if the above reaction takes place at a very low pressure, the temperature remaining at 25° ,



The problem underlying all biological oxidations is to explain the fact that substances such as carbohydrates, amino-acids, proteins, etc., when in contact with the living cells become easily oxidised even at ordinary temperatures, whilst in other circumstances they are perfectly stable to molecular oxygen. Obviously the cell may be exerting its influence on either of the components of the system; it may render the oxidisable substance so unstable that it reacts spontaneously with oxygen, or it may so activate oxygen that it becomes capable of reacting with the stable molecules of the oxidisable material; alternatively both actions may be at work.

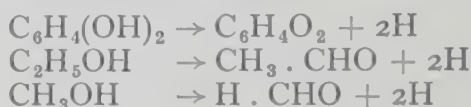
Methods used for the study of biological oxidations

Any chemical reaction involving a change of gas pressure can be studied manometrically. The theory and practice of manometry

is described in excellent monographs^{1, 2} to which the reader is referred, and the use of these methods will be taken for granted. As the overall effect of all aerobic oxidations involves the uptake of O_2 and in the majority of cases the output of CO_2 , manometric methods are uniquely adapted to their study.

In the case of intact cells, however, it is only the overall effect which can be so studied whilst intermediate stages demand the aid of additional methods. These intermediate happenings do not actually involve O_2 but consist of a series of anaerobic transfers of hydrogen.

The view that biological oxidation consists in the transfer of hydrogen to some spontaneously reducible substance was first advanced and elaborated by Wieland, who, concentrating on the intermediate processes, ignored the final step by which O_2 becomes involved. He was led to this view by the observation that many organic substances become anaerobically oxidised in the presence of finely divided metal catalysts (platinum or palladium black) by loss of hydrogen, which is absorbed or loosely combined with the metal and recoverable from it. Thus :

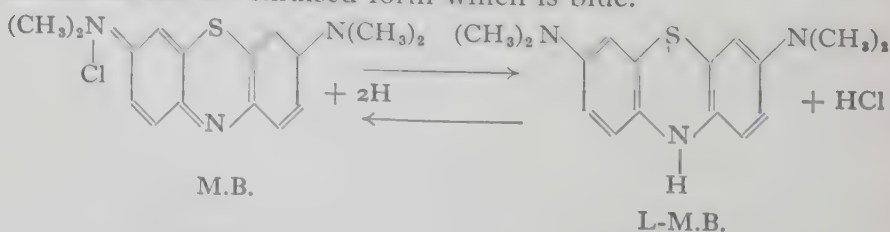


Glucose gives $CO_2 + 2H$.

Cases in which oxidation involves the entry of oxygen into the molecule were explained on this hypothesis as due to the intermediate formation of a hydrate and its subsequent dehydrogenation ; thus acetaldehyde was regarded as being oxidised through its hydrate to acetic acid, carbon monoxide through formic acid to carbon dioxide.

Use of the methylene blue technique

The study of much of the catalytic transfer of hydrogen ("dehydrogenation") by animal and plant tissues and by bacteria has been carried out by the use of methylene blue and other reducible dyes as hydrogen acceptors. The value of methylene blue is due to the fact that it exists in a reduced form which is colourless and an oxidised form which is blue.



¹ Dixon, 1943.

² Umbreit *et al.*, 1945.

The reduced form in the presence of oxygen is autoxidisable (apart from any catalyst), two atoms of hydrogen per molecule being transferred to molecular oxygen. By the use of the well-known technique of Thunberg it can rapidly be determined whether a tissue or enzyme preparation can effect the transfer of hydrogen from any substance, provided methylene blue is not toxic to the enzyme or cell. This method consists in placing in a vacuum tube a convenient quantity of the substance in solution with buffer of the necessary pH and the enzyme preparation; the tube is then evacuated and kept at a suitable temperature (usually about 40°); dehydrogenation of the substance is indicated by the change of the methylene blue to the leuco form. All necessary timed controls must be done to ensure that the process is enzymic (boiled control) and due to the substance in question and not some oxidisable substance introduced with the enzyme preparation (experiment omitting oxidisable substance). This technique was first applied extensively to bacteria by Quastel and Whetham.¹ The bacteria used were grown in pure culture in broth, centrifuged and washed and suspended in water or saline so as to form a thick suspension, and aerated for 1 hour to remove any easily oxidisable cell constituents. Such a suspension was described by the authors as "resting bacteria," indicating that in the conditions of the experiment multiplication does not occur. Provided that the experiment does not last longer than 2 to 3 hours this supposition is justified.^{2, 3} The number of substances which can be dehydrogenated by bacteria in this way is enormous and vastly exceeds those attackable by plant and animal tissues. This is perhaps not surprising considering the great variety of bacterial species and the divergence of their metabolic types, but even one species—e.g. *Esch. coli*—can attack a large number of substrates; these include lower members of the fatty acid series, dicarboxylic, hydroxy- and amino-acids, polyhydric alcohols and sugars. Many of the dehydrogenases of bacteria are found also in animal tissues, such, for example, as lactic and succinic dehydrogenases; others, such as formic dehydrogenase and hydrogenase, are peculiar to bacteria; the former catalyses the reaction $\text{HCOOH} + \text{A} = \text{CO}_2 + \text{AH}_2$ (where A represents the oxidised form of any hydrogen acceptor);^{4, 5} the latter catalyses the reaction $\text{H}_2 + \text{A} = \text{AH}_2$ and thus effects reductions by means of gaseous hydrogen.⁶

The use of this technique with washed intact cells, though giving only limited information, is yet exceedingly useful in preliminary studies on bacterial metabolism as indicating what mole-

¹ Quastel & Whetham, 1925 (1)

² Studdiford & Woodbridge, 1931.

³ Stickland, 1929.

⁴ Cook & Stephenson, 1928.

⁵ Quastel & Whetham, 1925 (1), (2).

⁶ Stephenson & Stickland, 1931 (1).

cules of a substrate are utilisable by the organism in question. For example, a large number of heterotrophic bacteria attack *d*-glucose readily by this method whilst failing to attack rarer sugars, whilst others like *Cl. sporogenes* attack none of the common sugars or organic acids but readily dehydrogenate certain amino-acids¹ (see p. 125).

In this connection it is important to take care that the substrate used is uncontaminated with other sugars or oxidisable compounds. Thus 1 ml. 1/5000 methylene blue (app. *M*, 6000) is equivalent to approximately 0.03 mg. glucose, assuming 1 mol. glucose donates 2 atoms of hydrogen; if 1 ml. 0.1 *M* galactose is used as substrate a contamination of the galactose with 0.05% glucose would suffice to effect the reduction of the methylene blue, the galactose being completely inactive. This is calculated on the assumption that 1 mol. methylene blue is completely reduced by 1 mol. glucose; actually 1 mol. glucose in the presence of *Esch. coli* reduces between 2 and 3 mols. of methylene blue. The theory underlying this technique will be discussed later (see p. 38).

The disruption of the bacterial cell

The intimate study of bacterial oxidations cannot be carried out on intact cells but involves the preparation of cell-free enzymes and coenzymes. The smashing of the bacterial cell is a matter of greater difficulty than the lysing of animal tissues and of larger microbial cells like yeasts. Three methods have so far been used for this purpose: (1) the crushing mill;² this apparatus was one of the first in the field and by its use enabled studies on bacterial cell-free enzymes to be carried out; equally good results have now been achieved by (2) supersonic sound waves; (3) grinding with powdered glass;³ (4) shaking with small graded glass balls.^{4, 5}

If cells treated by these methods are examined microscopically they are found to consist of a small proportion of apparently intact cells, some empty sheaths ("ghosts"), a number of cells with a rat-bitten appearance, due to damage of the cell membrane, and a large amount of structureless debris. If this material is spun down at about 3000 r.p.m. a supernatant is obtained which after dilution with an equal volume of water can be centrifuged at about 11,000 r.p.m. to give a transparent cell-free liquid. After dialysis against running water the dialysable constituents of the cell-juice are removed and the preparation consists mainly of proteins. These may be separated by fractional precipitation with ammonium sulphate and by other means.

In some cases these dehydrogenases can be separated from the

¹ Stickland, 1934.

² Booth & Green, 1938.

³ Wiggert *et al.*, 1940.

⁴ Curran & Evans, 1942.

⁵ Kalnetsky & Werkman, 1943.

cells by allowing the latter to autolyse, centrifuging the cell residue, and removing soluble cell constituents by dialysis. This simple procedure succeeds with lactic dehydrogenase of *Esch. coli* and the gonococcus^{1, 2, 3} and is due to the fact that this enzyme survives autolysis.

Components of the oxidising system

The bacterial cell has now been treated by rupture, separation into solid and liquid, and the latter into dialysable and non-dialysable fractions. It now remains to be seen what part of the oxidising mechanism of the cell has survived this treatment and in what fractions its components lie.

The lactic dehydrogenase (acting also on α -hydroxybutyric acid), obtained by any of the above methods from either *Esch. coli* or gonococcus, actively reduces methylene blue but fails to take up O_2 in the manometer, that is, the enzyme activating the transfer of $2H$ to methylene blue is present but that part of the system transferring $2H$ to O_2 is absent. In the presence of methylene blue, however, O_2 is taken up due to the reoxidation of *leuco* methylene blue by O_2 ; this is of course an artificial carrier and plays no part *in vivo*.

Many dehydrogenases, however, require further units for the completion of their action. The ethyl alcohol dehydrogenase from *Esch. coli*, for example, cannot transfer $2H$ direct to methylene blue but requires the mediation of a carrier which is present in the original cell but is lost during dialysis.⁴ This is the coenzyme I (or cozymase), i.e. adeninepyridine dinucleotide.^{5, 6, 7, 8} This compound exists in the oxidised and reduced states. The former is required to transfer the $2H$ from the reduced dehydrogenase but it requires a second enzyme for its own reoxidation; this is a flavoprotein, diaphorase,^{9, 10} which is removed from the dialysed enzyme system by acetone treatment.¹¹ This enzyme specifically catalyses the transfer of $2H$ from coenzyme I; its prosthetic group is flavinadenine dinucleotide and it has a wide distribution corresponding to its indispensable role in metabolism. With animal tissues there exists a second coenzyme (II), triphospho-adeninepyridinedinucleotide, which is reduced by a different range of dehydrogenases from those working with coenzyme I, but is itself oxidised by diaphorase and therefore subsequently transfers the $2H$ via the same path as coenzyme I. This compound is present in red blood cells and has not yet been found in bacteria; nor does it react with any of the bacterial dehydrogenases

¹ Stephenson, 1928. ² Barron & Hastings, 1933. ³ Still, 1940. ⁴ Ibid.

⁵ Lohr & Ventin, 1935.

⁶ Warburg & Christian, 1933 (1), (2).

⁷ Lohr & Schlenk, 1937.

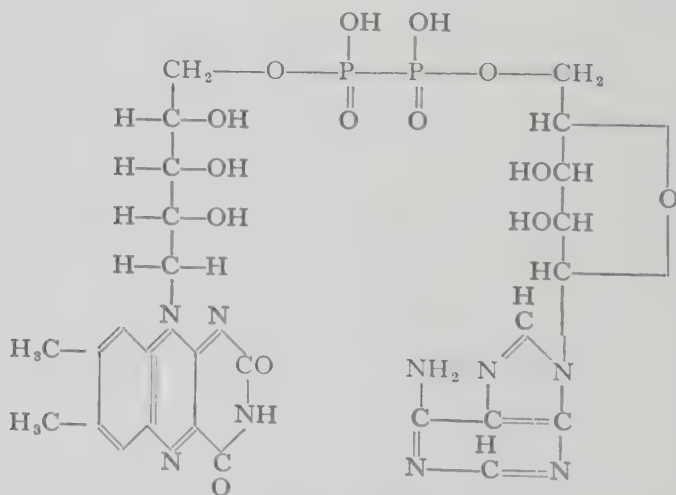
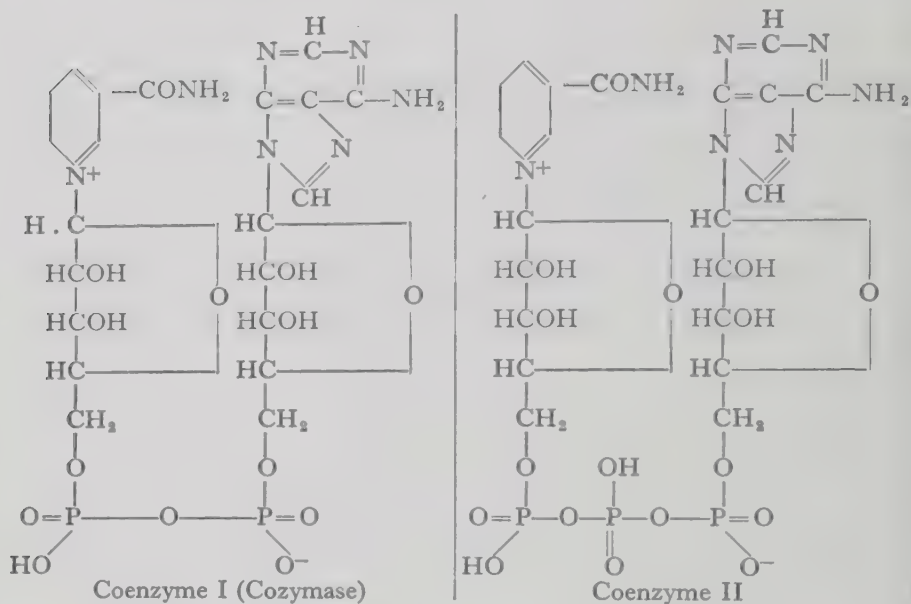
⁸ Warburg, Christian & Griese, 1935.

⁹ Dewar & Green, 1938.

¹⁰ Straub, 1939.

¹¹ Still, 1940.

so far separated from bacteria. Some flavoprotein enzymes are spontaneously autoxidisable; where these occur they complete the oxidation of the substrate by molecular oxygen. The flavin-adenine dinucleotide present in most aerobic cells and functioning as the reoxidising agent for the adeninepyridinedinucleotide is not of this type and reacts with O_2 only through another series of enzymes.



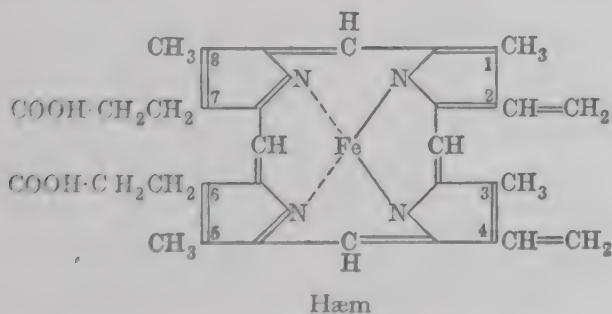
Prosthetic group of diaphorase
Flavinadenine dinucleotide

The iron-porphyrin enzymes

The final stages of aerobic oxidation, viz. the transfer of $2H$ to O_2 , are catalysed by the cytochrome system which is composed of several iron-porphyrin compounds. Two other enzymes which play an essential part in biological oxidations, catalase and peroxidase, also belong to the group of iron-porphyrin derivatives.

The iron-porphyrin compounds constitute together with magnesium porphyrins (chlorophyll) and copper porphyrins (e.g. turacin) the large group of metal porphyrins. Porphyrins as such are substances derived from a structure composed of four pyrrol rings connected together by four CH -links. They are classified according to the nature of the side groups (methyl, ethyl, vinyl, etc.) attached to the porphyrin nucleus in positions 1 to 8. However, out of the many synthetically prepared porphyrins there are only a few which occur in nature and the most commonly found is protoporphyrin. This is generally found in combination with iron. According to the valency of the iron, the iron-porphyrin is designated as hæm (divalent Fe) or hæmatin (trivalent Fe). Both hæm and hæmatin occur in numerous compounds in combination with proteins. Some of these compounds are biologically active only in the hæm form (e.g. hæmoglobin) or in the hæmatin form (e.g. peroxidase), but others act by being continuously oxidised and reduced, i.e. both in the hæm and hæmatin form. Typical representatives of this kind are the cytochromes.

It may be mentioned here that not all biologically active metal compounds belong to the porphyrin derivatives. Thus polyphenol oxidase, the enzyme responsible for the darkening phenomena in many plants, is a copper protein in which Cu is linked to the protein molecule directly and not through a porphyrin.



The porphyrins and their compounds are characterised by strong absorption spectra; which serve to identify them and to provide easily observable signs of chemical changes.

The principal enzymes of this group known to be present in bacteria may now be considered.

Cytochromes

The cells of all aerobes—plant, animal and microbial—contain a series of iron-porphyrin proteins with strong catalytic properties and well-marked absorption spectra known collectively as cytochrome. Cytochrome was first described from spectroscopic observation of animal tissue by MacMunn,¹ but he was unable to convince his contemporaries of the importance of his discovery

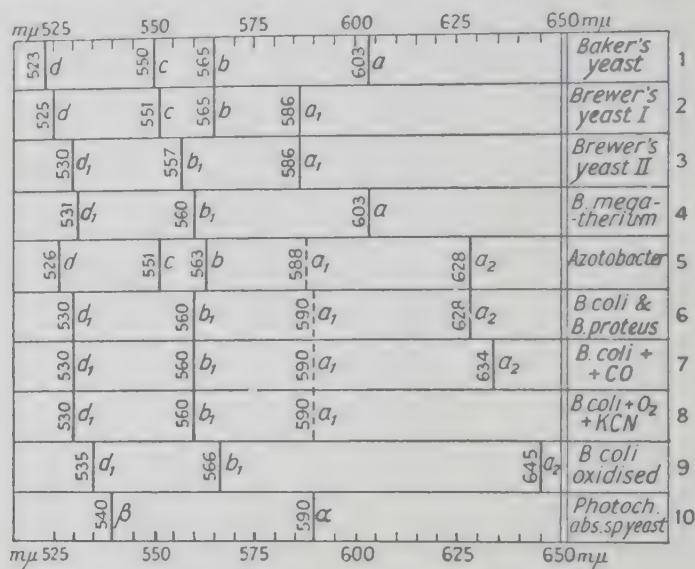


FIG. 1

which formed a lost island in knowledge till rediscovered by Keilin in 1925.² Cytochrome is absent from all strict anaerobes (*Clostridia*) so far examined and from some facultative anaerobes, e.g. many lactic organisms. The amount present in facultative anaerobes varies according to conditions of growth; anaerobic conditions and adaptation to cyanide resulting in a decrease in the amount formed. Cytochrome exists in oxidised and reduced forms. The latter has a well-marked spectrum with four bands occupying approximately the positions *a*, 6045 Å; *b*, 5662 Å; *c*, 5500 Å; *d*, 5205 Å. The first three bands (*a*, *b* and *c*) represent the α -bands of three hæmochromogen-like components of cytochrome known as cytochrome *a*, *b* and *c*; the fourth band (*d*) represents the fused β -bands of all cytochromes; in addition free hæmatin is also found.

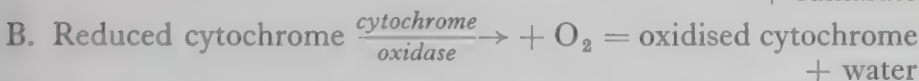
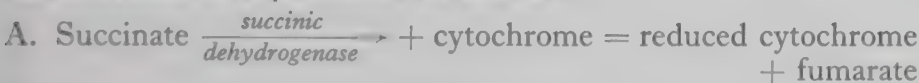
By the use of the microspectroscope the cytochrome of baker's yeast or aerobic bacteria may be seen to undergo alternate oxidation on shaking with air and reduction on standing or, more

¹ MacMunn, 1886.² Keilin, 1925.

quickly, on bubbling with nitrogen; the suspension will remain permanently in the reduced condition on poisoning with cyanide and in the oxidised condition by the action of narcotics.

It was originally thought that the agents reducing cytochrome were the dehydrogenases of the cell, since all agents poisoning these enzymes prevented cytochrome reduction. Cytochrome *c* has, however, now been prepared in a pure state apart from the cell,¹ thus permitting of its study in a more precise manner. The *Eo* at pH 7.0 of the system reduced cytochrome \rightleftharpoons oxidised cytochrome is -0.12 v.;² it might therefore be supposed that the reduced form of any substance with a more negative potential with its dehydrogenase would reduce cytochrome; this, however, is not the case, the only ones so far found capable of functioning in this way being the lactic dehydrogenase of yeast, the succinic dehydrogenase and the α -glycerophosphate dehydrogenase of animal tissues.³ The succinic dehydrogenase of bacteria has not been obtained apart from the cell so cannot yet be tested; the behaviour of the lactic dehydrogenase of *Bact. coli* is not certain. Although substances capable of direct oxidation through the cytochrome system are few, other substances may be brought into the system by a preliminary process of oxidoreduction; this is made clear on p. 26.

Cytochrome *c* does not react directly with oxygen; the reduced form has so far only been oxidised by the enzyme cytochrome oxidase with which it is closely associated in the cell. The system can therefore be represented as follows:



A is strongly inhibited by heat and narcotics, B by heat, cyanide and carbon monoxide. This accounts for cytochrome in the cell remaining in the reduced condition when poisoned with cyanide and in the oxidised condition when poisoned with narcotics, neither of these poisons acting on the cytochrome itself. The cytochrome oxidase is almost certainly identical with the oxygen-transporting enzyme or *Atmungsferment* studied by Warburg. The former has been prepared from heart muscle,^{4, 5} and in this condition oxidises only cytochrome *c*, the rates of oxygen uptake being approximately the same as that due to the aerobic respiration of a corresponding amount of intact tissue; the reoxidation of cytochrome is inhibited by $-\text{CN}$ and CO , H_2S and NaN_3 , the CO

¹ Keilin & Hartree, 1937 (1).

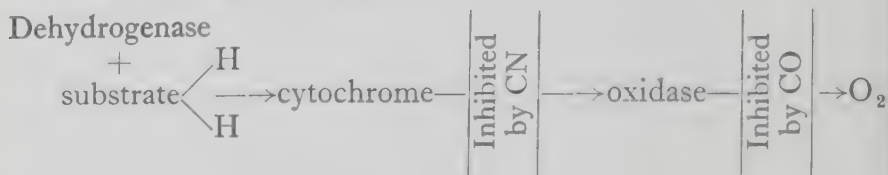
² Green, 1934.

³ Ogsten & Green, 1935.

⁴ Keilin & Hartree, 1938 (1).

⁵ Warburg, 1926.

inhibition being reversed by exposure to light. Warburg¹ has pointed out that the inhibitions by $-\text{CN}$ and CO are evidence that the enzyme is an organic iron compound since it is a property of iron in organic combination to combine with both these substances. Thus trivalent iron combines with $-\text{CN}$; this is shown by the fact that cyanide inhibition is independent of oxygen pressure; $-\text{CN}$ therefore inhibits the reduction of the enzyme. CO , like O_2 , combines with the reduced (divalent) form of the enzyme and the action of these two is competitive; CO therefore inhibits the oxidation of the enzyme and the action is reversible by light. The transfer of hydrogen is represented thus:



The evidence for the existence of an iron-containing substance with these relations to $-\text{CN}$ and CO as given by Warburg and his colleagues has been based entirely on indirect methods. Kubowitz and Haas² have thus demonstrated by an indirect method the existence in certain aerobic cells (e.g. *Acetobacter pasteurianum* and baker's yeast) of a substance with a distinct absorption spectrum having the properties of the oxidase. For this spectroscopic study use is made of the fact that the CO inhibition is reversible by exposure to light; this implies that the enzyme forms a CO -compound decomposed by light. For the light to act in this way it must be absorbed, hence the quanta of light absorbed at any given wavelength can be measured by the decomposition of the CO -enzyme complex, which in its turn can be measured by the oxygen uptake of the cell. Thus assuming that the incident quanta of light energy equal the quanta absorbed, and that the intensity of the illumination is known, the light absorption coefficient is given by the equation

$$\frac{B_1}{B_2} = \frac{W_1}{W_2} \cdot \frac{l_2}{l_1}$$

where W_1 and W_2 are the rates of oxidation per unit of material at wavelengths 1 and 2 respectively. Fig. 2 gives the absorption spectrum obtained in this way from suspensions of the aerobic organism *Acetobacter pasteurianum*. Here the absorption at $436 \text{ m}\mu = 100$, this being approximately the position of maximum absorption. The bands on the left of this are subsidiary bands in the ultra-violet, those on the right are the x -band at $590 \text{ m}\mu$, the

¹ Warburg, 1926.

² Kubowitz & Haas, 1932.

β -band at $540\text{ m}\mu$ and the β' -band at $524\text{ m}\mu$. The spectrum obtained for *A. pasteurianum* approximates very closely to that from other aerobic cells such as baker's yeast. In thick suspensions of *A. pasteurianum*¹ a band at $589\text{ m}\mu$ has been observed which disappears on oxidation but is sharpened by $-\text{CN}$. If treated with $-\text{CN}$ and shaken with oxygen it disappears while the cytochrome bands are unchanged; on saturation with CO it shifts to $593\text{ m}\mu$. This band therefore behaves as would be expected if it belonged to the oxidising enzyme.

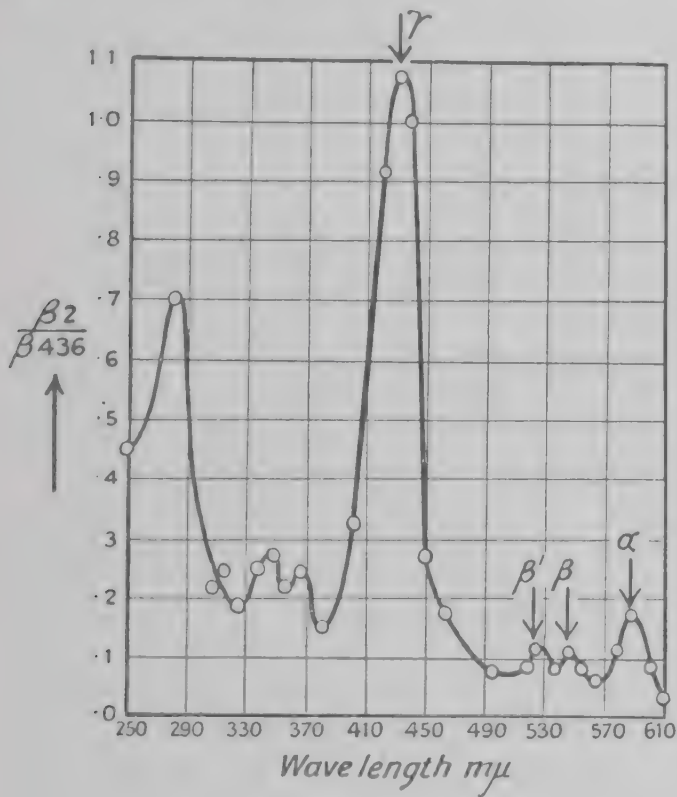


FIG. 2

More recent studies² have provided direct spectroscopic evidence that cytochrome oxidase is an iron compound with many of the properties of a hæmochromogen chemically related to the other cytochromes. The existence of this compound was for long unrecognised because in the oxidised state its bands are invisible and in the reduced (hæmochromogen) state they coincide with those of cytochrome *a*. The new compound has been named by its discoverers *a*₃ in order to distinguish it from cytochrome *a* as well as from *a*₁ ($589\text{ m}\mu$) and *a*₂ ($630\text{ m}\mu$) occurring in some

¹ Warburg & Negelein, 1933.

² Keilin & Hartree, 1939.

bacteria. The evidence in favour of a_3 being identical with cytochrome oxidase is as follows:

1. It is thermolabile.
2. It is affected by chemical treatment and agents such as acids and alkalis, acetone, alcohol, freezing and drying in the same way as is cytochrome oxidase.
3. It is autoxidisable and when in the reduced form combines with CO, forming a compound having an α -band at $590\text{ m}\mu$ corresponding to the CO band of Warburg's *Atmungsferment* and a γ -band at $432\text{ m}\mu$.
4. It combines with $-\text{CN}$ both in the divalent and trivalent state, forming two different compounds of which the former (CN-hæmochromogen) is easily autoxidisable while the latter (CN-parahæmatin) is stabilised in the oxidised condition and cannot be then easily reduced.
5. In the divalent state cytochrome a_3 combines with the following well-known inhibitors of cytochrome oxidase: H_2S , NaN_3 and NH_2OH .
6. a_3 is reduced by the same biological systems as is cytochrome oxidase.

Cytochrome and cytochrome oxidase in bacteria

The cytochrome in bacteria corresponds roughly to their respiratory character. Many highly aerobic organisms have a complete cytochrome spectrum, whilst in many facultative anaerobes one or more components (usually c) is missing. All members of the *Clostridia* so far reported have no cytochrome components and the same is true of certain streptococci (see Table 1).

The presence of cytochrome oxidase is shown by the effect of CO on oxidations by the cell. For this purpose the oxidation of p -phenylenediamine can be measured manometrically; this is oxidised by cytochrome which in its turn is reoxidised by the oxidase. The inhibition of this oxidation by CO and the reversal of the inhibition by light are characteristic of the cytochrome oxidase. Where the oxidation of the p -phenylenediamine does not exhibit this behaviour towards CO and light (as reported for some species of bacteria¹) the cytochrome oxidase is not an active component of the system. Cytochrome oxidase effects only the oxidation of cytochrome; the oxidation of p -phenylenediamine (and of biological hydrogen donors) occurs only through cytochrome as an intermediary.²

If a thick paste made by centrifuging an aerobic organism such as *Bac. subtilis* or a highly aerobic yeast be examined with the microspectroscope, the four bands of cytochrome are generally not at first visible since the absorption spectra are characteristic of the reduced forms of the cytochromes. If now glucose, succinate, or any substance for which the organism possesses a dehydrogenase, be added the four bands appear; on shaking with air they

¹ Frei Riedmuller & Almasy, 1934.

² Keilin & Hartree, 1938 (2).

TABLE 1
DISTRIBUTION OF CYTOCHROME IN BACTERIA

Species and Strains	Absorption bands of reduced cytochrome			
Aerobes				
<i>B. subtilis</i>	a	b	c	d
<i>B. mesentericus vulgatus</i>	a	b	c	d
<i>B. anthracis</i>	a	b	c	d
<i>B. mycoides</i>	o	b	o	d
<i>B. tuberculosis</i>	a	b	c	d
<i>Azotobacter chroococcum</i>	o	b	c	d
" <i>vinelandii</i>	o	b	c	d
<i>B. xylinum</i>	o	o	c	d
<i>B. pasteurianum</i>	o	o	c	d
<i>Sarc. aurantiaca</i>	a	b	o	d
" <i>lutea</i>	a	b	c	d
<i>Staph. aureus</i>	a	b	o	d
" <i>albus</i>	a	b	o	d
" <i>citreus</i>	a	b	o	d
<i>V. cholerae</i>	a	b	c	d
<i>Gonococcus</i>	o	o	c	d
<i>Meningococcus</i>	o	o	c	d
Facultative anaerobes				
<i>B. pyocyaneus</i>	a	b	c	d
<i>B. fluorescens liquefaciens</i>	a	b	c	d
<i>B. denitrificans</i>	a	b	c	d
<i>B. proteus vulgaris</i>	o	b	c	d
<i>B. coli communis</i>	o	b	o	d
<i>B. coli communior</i>	o	b	o	d
<i>B. typhosum</i>	o	b	o	d
<i>B. paratyphosum A</i>	o	b	o	d
<i>B. paratyphosum B</i>	o	b	o	d
<i>B. dysenteriae shiga</i>	o	b	o	d
<i>B. dysenteriae flexner</i>	o	b	o	d
<i>Pneumococcus I</i>	a	b	o	d
<i>Str. acid lactici</i>	o	o	o	o
<i>B. delbrückii</i>	o	o	o	o
<i>B. acidophilus</i>	o	o	o	o
Strict anaerobes				
<i>Cl. tetanum</i>	o	o	o	o
<i>Cl. sporogenes</i>	o	o	o	o
<i>Cl. welchii</i>	o	o	o	o
<i>Cl. putrificum</i>	o	o	o	o

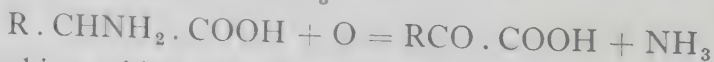
again disappear and this alternate reduction and oxidation can be repeated indefinitely. If now a drop of cyanide is added to the preparation the reoxidation is permanently inhibited and the bands no longer disappear on shaking with air. If instead an inhibitor for the dehydrogenases—for example, narcotics—is used the cytochromes remain in the oxidised state, i.e. no bands are visible; it can be shown, however, that the cytochromes are still intact as they reappear on the addition of a chemical reducing agent such as

sodium hydrosulphite. The naturally occurring reducing agent for cytochromes *a* and *b*—less certainly for *c*—is the flavoprotein diaphorase, which thus acts as the link between cytochrome and those dehydrogenases working through coenzyme I. The majority of the dehydrogenases active in bacteria are of this type. The cytochromes in their case are oxidised by cytochrome oxidase which transfers hydrogen to molecular oxygen, forming water.

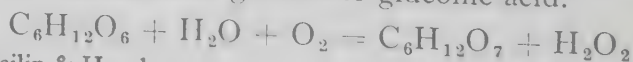
The facultative anaerobe, *Bact. coli*, when grown aerobically takes up oxygen by means of a system which is reversibly inhibited by $-\text{CN}$, H_2S , azide and CO , thus resembling typical aerobic cells; the inhibition by CO is, however, not reversed by light, indicating that cytochrome oxidase is not involved. Spectroscopic examination shows that the band a_3 (cytochrome oxidase) is absent, as is also cytochrome *c*.¹ This is in agreement with the fact that crushed cells of this organism are unable to oxidise cytochrome *c* supplied from another source or to oxidise *p*-phenylenediamine either in the presence or absence of added cytochrome *c*. This system is further differentiated by the absence of cytochrome *a*, the band of which is replaced by a hardly perceptible band a_2 at 628 $m\mu$ similar to a_2 of *Azotobacter*. The typical band *b* is also absent and is replaced by b_2 at 560 $m\mu$. The band a_2 belongs to an autoxidisable compound which reacts with $-\text{CN}$, when the band disappears, and with CO , when it moves to 634 $m\mu$. The general conclusion derived from these observations seems to be that in this organism cytochrome b_1 is reduced by the dehydrogenases and oxidised by a_2 , which is an autoxidisable component fulfilling the function of cytochrome oxidase. a_2 appears to be identical with the a_2 of the aerobic organism *Azotobacter*.

Direct-oxidising enzymes

Besides the dehydrogenases which pass the 2H through the coenzyme I-cytochrome system to O_2 , there exist a few enzymes which appear to transfer 2H direct from the substrate to O_2 , forming H_2O_2 or in some cases H_2O . Among these is the oxidase separated from *Proteus vulgaris* oxidising a number of α -amino-acids to the keto-acids + NH_3 .²



the xanthine oxidase of milk and liver (acting also on aldehydes), the glucose oxidase of *A. niger* first described by Müller³ and probably identical with notatin.⁴ The last-mentioned oxidase catalyses the oxidation of glucose to gluconic acid.



¹ Keilin & Harpley, 1941.

³ Müller, 1928.

² Stumpf & Green, 1944.

⁴ Coulthard *et al.*, 1942.

Such oxidases may function in certain facultative anaerobes (certain streptococci and some lactic bacteria) which, though living mainly by anaerobic mechanisms, do, when strongly aerated, produce H_2O_2 , though possessing no cytochrome, catalase or peroxidase.

Dehydrogenases reducing cytochrome c direct

In the case of animal tissues certain variations on the above scheme exist. There are a few dehydrogenases—succinic dehydrogenase and α -glycerol phosphate dehydrogenase—which appear to reduce cytochrome *c* directly without the mediation of coenzyme I or diaphorase; so far these enzymes have not been shown to have any prosthetic group attached to the enzyme protein which might replace the flavin moiety of diaphorase. The succinic dehydrogenase of heart tissue has, however, recently been split into two components, the dehydrogenase proper and a component transferring $2H$ to cytochrome *c*. The succinic dehydrogenase of bacteria has not so far been separated from the cell so it is uncertain whether it behaves in the same way as the corresponding enzyme from animal sources, but the formic dehydrogenase of *Esch. coli* certainly belongs to this category.¹ Table 2 gives an example of each type of dehydrogenase.

TABLE 2

Dehydrogenase reacting with			
Coenzyme I and the cytochrome system	Coenzyme II and the cytochrome system	Cytochrome direct	O ₂ direct
Triosephosphate (<i>E. coli</i>)	Hexosemonophosphate (animal)	Succinic acid (animal) ²	Xanthine oxidase Aldehyde oxidase (milk and liver)
Ethylalcohol (<i>E. coli</i>)	Glycerolphosphate (animal)		Glucose oxidase
Lactic acid (yeast) β -hydroxybutyric acid (animal)	—	Formic acid	<i>L</i> - α -amino-acid oxidase (<i>Proteus vulgaris</i>)
Malic acid (<i>E. coli</i>)	—	—	—

The polyphenol oxidase and laccase

A system analogous to the Warburg-Keilin system is important in some plants.³ This consists of an oxidase which oxidises certain *o*-dihydroxyphenols such as catechol, pyrogallol and *p*-creosol to *o*-quinones, which are in turn reduced by other substances

¹ Gale, 1939.

² See however Straub, 1942.

³ Onslow, 1931.

such as ascorbic acid and reduced coenzyme. The hydrogen-transferring system may therefore be represented thus :



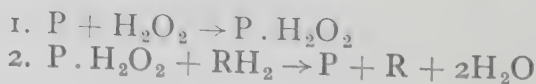
Catechol and catechol oxidase therefore replace cytochrome and cytochrome oxidase in the other system. Catechol oxidase has been separated from mushrooms in a highly purified state¹ and is a copper protein compound with a Cu content of 0.30% and is of great activity, 1 μg . Cu transferring 6000 μl . O_2 per minute at 20°; giving a Q_{O_2} of 1,600,000. It has not so far been reported in bacteria.

In addition some plant tissues contain another enzyme, laccase,² catalysing also the oxidation of *para*-compounds such as hydroquinone and *p*-phenylenediamine and also ascorbic acid.

Peroxidase and catalase

In addition to the systems responsible for the main-line course of oxidation two subsidiary systems exist concerned with the utilisation or removal of the hydrogen peroxide formed by the primary system; these are peroxidase and catalase.

Peroxidase is an enzyme (or possibly a group of enzymes) characteristic of plants and found also in some animal material, catalysing the oxidation of certain substances by hydrogen peroxide. This enzyme has been prepared in a highly purified and very active condition ($Q_{\text{O}_2} = 2.5 \times 10^6$)* from horse radish and its structure and properties studied.³ It is a hæmatin compound with a characteristic absorption spectrum capable of forming two well-defined compounds with H_2O_2 ; in the presence of certain oxidisable substances the decomposition is very rapid and is accompanied by the oxidation of the substance. The action of peroxidase (P) with H_2O_2 and the oxidisable substance (R) can be represented thus :



These reactions are inhibited by —CN which combines with peroxidase. Examples of oxidations effected by peroxidase activity are *o*-diphenols to quinones, nitrite to nitrate, tyrosine and tryptophan to coloured products, reduced glutathione and ascorbic acid to their respective oxidised forms. The principal source of animal

¹ Keilin & Mann, 1938.

² Ibid., 1939, 1940.

³ Ibid., 1937.

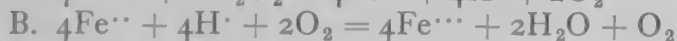
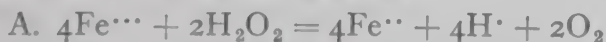
* Calculated from the purpurogalline number 1500.

peroxidase is milk; a peroxidase has also been described in mammalian lung and spleen.¹

Some confusion has arisen owing to the use of benzidine, guaiacum and *p*-phenylenediamine as tests for peroxidase in tissues. These substances are oxidised by peroxidase and H_2O_2 and such reactions are heat-labile, but they are also oxidised by many hæmatin compounds present in blood and tissues (including cytochrome *c*), and such oxidations, though quantitatively insignificant (and heat-stable), are liable to be confused with true peroxidase reactions when only qualitative tests are applied. Many peroxidases reported in bacteria are probably of this type.

Catalase is an enzyme related to peroxidase, more characteristic of animal tissues than of plants and present also in aerobes and in many facultative anaerobes. It has been separated in a high degree of activity ($Q_{\text{O}_2} = 8 \times 10^7$) from pig's liver² and found to be a hæmatin compound. It catalyses the decomposition of H_2O_2 into water and molecular oxygen and is regarded as a protective mechanism whereby the H_2O_2 formed in other oxidations is rendered innocuous.

The mechanism of the action of catalase has been elucidated³ by use of the purified preparation. Anaerobically the decomposition of H_2O_2 does not occur, and it can further be shown spectroscopically by using the azide and hydroxylamine compounds of catalase that H_2O_2 is the only substance which (anaerobically) reduces catalase iron. The aerobic action of catalase and H_2O_2 is therefore represented by an anaerobic reduction of catalase iron by H_2O_2 and an aerobic oxidation by O_2 .



A is inhibited by KCN, H_2S and $\text{C}_2\text{H}_5\text{OH}$; B by azide, hydrazine and hydroxylamine.

Thus in peroxidase and catalase we have two enzymes with many points in common but quite distinct types of activity; their common properties being attributable to their common hæmatin nucleus and their differences to the fact that the attached protein molecules are different.

Recent investigations, however,⁴ throw doubt on the intracellular protective action of catalase in decomposing H_2O_2 to $\text{O}_2 + \text{H}_2\text{O}$. Mammalian red blood cells are rich in catalase and when H_2O_2 is added to them it is violently decomposed, the oxyhæmoglobin remaining unchanged. It therefore appears that the catalase has protected the hæmoglobin from oxidation to

¹ Bancroft & Elliott, 1934.

² Ibid., 1937 (2).

³ Keilin & Hartree, 1936.

⁴ Ibid., 1945.

hæmatin by H_2O_2 . If, however, the H_2O_2 is delivered to the cell in low concentration by the interaction of an enzyme system—for example, glucose oxidase and glucose—it is then found that the hæmoglobin is rapidly oxidised to methæmoglobin; that is to say that catalase protects hæmoglobin from H_2O_2 at high concentrations but not at low ones. This apparent contradiction is explained by the fact that at high concentrations of H_2O_2 , O_2 is produced sufficiently rapidly to oxygenate hæmoglobin to oxy-hæmoglobin; when in the oxygenated (as opposed to the oxidised) state the hæm (ferroprotoporphyrin) does not react with H_2O_2 . When, however, the H_2O_2 is produced at very low concentrations the tension of the O_2 liberated by catalase is insufficient to push the reaction $\text{Hb} + \text{O}_2 \rightleftharpoons \text{HbO}_2$ to the right and the hæmoglobin is in the unoxygenated state and susceptible to oxidation to hæmatin by the low concentrations of H_2O_2 . In oxidising systems and cells where H_2O_2 is produced the latter can be broken down in three ways:

1. By catalytic decomposition $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$.
2. By certain substances, notably pyruvate, which are spontaneously oxidisable by H_2O_2 .



This reaction occurs in the presence of H_2O_2 only and it does not require enzyme catalyses.

3. By secondary oxidations catalysed by catalase in virtue of its peroxidase function. The oxidations at present shown to occur most readily in this way are methanol, ethanol and *n*-propanol to their respective aldehydes. Higher alcohols are but little attacked, nor are the sugars, lactate and malate.

The role of hydrogen peroxide

The majority of aerobes and facultative anaerobes possess catalase by which means the H_2O_2 produced in aerobic oxidations is decomposed before it attains a sufficient concentration to become toxic to the organism. Hydrogen peroxide can, however, be demonstrated in some bacteria as a result of aerobic oxidation; chief among these are the *Pneumococci*,¹ *Streptococci* and two lactic-acid producing bacilli, *B. bulgaricus* and *B. acidophilus*.^{2, 3, 4} Some organisms are devoid of catalase but have not been shown to produce H_2O_2 , viz. *B. dysenteriae shiga*, certain *Streptococci*, and some other strains.

A special case of an organism lacking catalase is that of *Acetobacter peroxydans*. This aerobe, however, produces no demon-

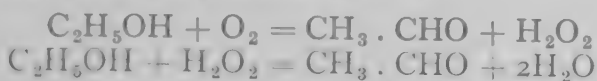
¹ M'Leod & Gordon, 1922.

² Ibid.

³ Rywosch & Rywosch, 1907.

⁴ Callow, 1923; M'Leod & Gordon, 1923 (1), (2); Avery & Neill, 1924.

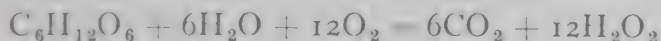
strable H_2O_2 , but the oxidising system differs from the lactic bacteria just mentioned in being cyanide sensitive. Hydrogen peroxide at M 250 is inhibitory to oxidation but at M 800 acts as hydrogen acceptor at a high velocity ($Q_{\text{O}_2} = 500$). Acetaldehyde anaerobically gives only traces of acetic acid, showing that aldehyde mutase is almost entirely lacking, hence the oxidation of alcohol is regarded as proceeding thus:



It appears that we have here a case of an organism using a peroxidase system as its main respiratory mechanism; *p*-phenylenediamine is oxidised by H_2O_2 and in the presence of the organism probably by the same peroxidase. Spectroscopic examination shows an absorption at \AA 5526 to 5556.

Hydrogen also is oxidised by oxygen and by H_2O_2 , but the former oxidation soon comes to a standstill. The mechanism here is a little obscure; aerobically hydrogen does not react with H_2O_2 , possibly on account of the high affinity of oxygen for the enzyme.¹

In the case of the lactic bacteria the system seems unadapted to aerobic life; here during the early stages of the oxidation of glucose the H_2O_2 accumulating is roughly proportional to the oxygen taken up:



This suggests that the H_2O_2 is not used for any secondary oxidation; the absence of peroxidase is confirmed by the fact that the oxidation is unaffected by CO and $-\text{CN}$.² The case of the pneumococcus is again different. Here the H_2O_2 formed in the primary oxidation of glucose reacts with pyruvic acid which protects the enzyme systems in the same way as added catalase; it is not clear whether this effect is due to peroxidase.³

Glutathione

This substance was first isolated by Hopkins⁴ from yeast, mammalian muscle and liver in proportions of 0.1 to 0.15 g. per kg. It was at first believed to be a dipeptide, glutaminyl cysteine, but was later shown to be a tripeptide, glutaminylcysteinylglycine.⁵ From the biochemical standpoint the importance of glutathione lies in its possession of the $-\text{SH}$ group. In neutral or alkaline solution this is readily autoxidisable, two molecules of the

¹ Wieland & Pictor, 1936.

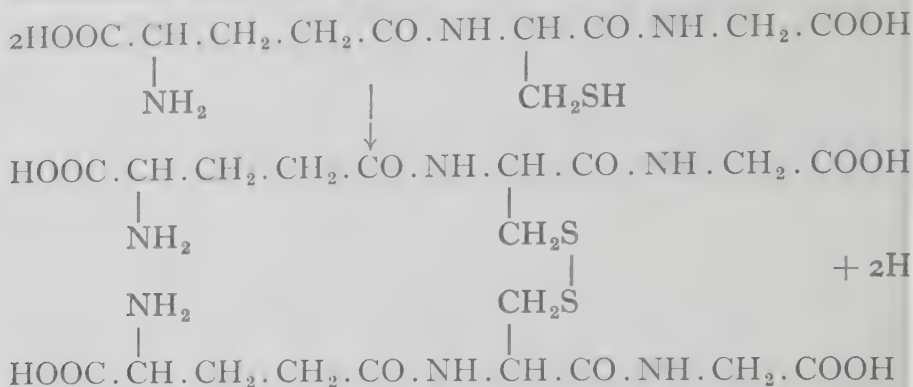
² Bertho & Glück, 1932.

³ Sevag, 1933.

⁴ Hopkins, 1921.

⁵ Ibid., 1929.

—SH form being oxidised to one of the —S—S— form; for convenience the former is written GSH and the latter GSSG.



Glutathione is an intracellular product of all actively growing animal and plant tissues and probably of many bacteria. Its presence in the reduced form is indicated by a nitroprusside reaction given by cells which have previously been disintegrated mechanically by hypertonic salt solutions, dilute acids or other means. To aqueous suspensions of such preparations ammonium sulphate is added to saturation followed by a small amount of nitroprusside and strong ammonia; glutathione is indicated by a pinkish magenta colour which quickly fades. Bacteria must be washed free from culture media before the test is performed, as a number of substances often present in media as a result of bacterial action, such as hydrogen sulphide and substances having the —SH group, as well as acetone, give a similar colour with nitroprusside. Glutathione has not so far been isolated from bacteria owing to the difficulty of obtaining sufficient material, but well-washed suspensions have been examined qualitatively.¹

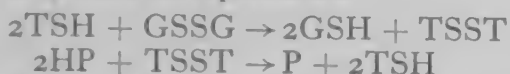
The physiological role of glutathione is very difficult to appraise. *In vitro* it catalyses the oxidation of sulphhydryl proteins. Muscle proteins prepared by thorough extraction of muscle with boiling water and drying with alcohol and ether (thus removing all soluble hydrogen donors and destroying enzymes) reduce GSSG to GSH at neutral pH, the GSH being oxidised aerobically to GSSG and again reduced to GSH by the washed muscle proteins. The transfer of hydrogen from the protein to GSSG and from the GSH to oxygen is independent of enzyme action, but the latter reaction is conditioned by the presence of iron complexes and inhibited by cyanide.^{2, 3} The oxidation of protein by GSSG appears to depend on the existence on the protein of insoluble substances giving the nitroprusside reaction known as fixed —SH (TSH).

¹ Callow & Robinson, 1925.

³ Meldrum & Dixon, 1930.

² Harrison, 1924.

If such material be incubated anaerobically with GSSG the hydrogen is transferred from the TSH to the GSSG; this in turn is oxidised aerobically, the total oxygen taken up being many times larger than the total —SH (both fixed and soluble) of the system. The oxidation may thus be represented



It is difficult to say whether this system is purely artificial or whether it also functions *in vivo*.

It appears that *in vivo* the reduction of glutathione can occur through some thermolabile dehydrogenase systems. This has been shown in the case of liver,^{1, 2} where glucose is the probable donator, and in intact red blood cells, where glucose, mannose, galactose and fructose all function.³ Glutathione may also function as an intracellular reducing agent; it has, for example, been shown that it re-reduces ascorbic acid after oxidation by the enzyme ascorbic oxidase *in vivo* and serves a similar function for intracellular enzymes or carriers which need to be kept in a reduced condition.

Another function performed by this compound is that of co-enzyme of glyoxalase;⁴ the mechanism of this action is not clear but appears to depend on the formation of a compound between methylglyoxal and GSH and its decomposition into GSH and lactic acid, both reactions being catalysed by glyoxalase.^{5, 6}

The incomplete oxidation of substrate

When using intact cells it has been found that aerobically the oxidation of many substrates is incomplete; thus in the case of washed suspensions of *Bact. coli* and *Bact. alcaligines* lactate, pyruvate and acetate are oxidised to $\frac{2}{3}$, $\frac{3}{5}$ and $\frac{3}{4}$ of completion respectively. In the case of *Bact. coli* glucose is oxidised to the same extent as lactate; formate, on the other hand, is oxidised to completion.⁷ No products of incomplete combustion were found. A similar state of affairs was found for the *Spirilla*⁸ with a number of different compounds. Giesberger advanced the theory that the incomplete oxidation is associated with assimilation; the CO_2 output — O_2 uptake of various compounds studied are in close agreement with this hypothesis. Thus Tables 3 and 4 give the experimental figures upon which they are based and show the extent of agreement with theory.

These observations were extended by Clifton,⁹ using *Ps. calcoaceticus*. Here it was found that with the normal organism the

¹ Hopkins & Elliott, 1931.

² Mann, 1932.

³ Meldrum, 1932.

⁴ Lohmann, 1932.

⁵ Jowett & Quastel, 1933.

⁶ Yamazoye, 1936.

⁷ Cook & Stephenson, 1928.

⁸ Giesberger, 1936.

⁹ Clifton, 1937.

oxidation of acetate and butyrate reaches about $\frac{2}{3}$ of completion yet when the cell is poisoned with sodium azide ($M/1600$) or with 2:4-dinitrophenol ($M/4000$) complete oxidation is obtained. These poisons therefore appear to inhibit the assimilatory process without greatly decreasing the rate of oxidation which proceeds to completion. In order to clinch this theory it remains to demonstrate an intracellular storage of carbohydrate, as has been shown for the alga *Prototheca zopfii*.¹ For further information along these lines the reader is referred to a review by Clifton.²

TABLE 3³

1. Acetic acid . . .	$C_2H_4O_2 + O_2 \rightarrow CO_2 + (CH_2O) + H_2O$
2. Propionic acid . . .	$2C_3H_6O_2 + 6O_2 \rightarrow 5CO_2 + (CH_2O) + 5H_2O$
3. Lactic acid . . .	$C_3H_6O_3 + 2O_2 \rightarrow 2CO_2 + (CH_2O) + 2H_2O$
4. Pyruvic acid . . .	$2C_3H_4O_3 + 3O_2 \rightarrow 4CO_2 + 2(CH_2O) + 2H_2O$
5. Succinic acid . . .	$2C_4H_6O_4 + 5O_2 \rightarrow 6CO_2 + 2(CH_2O) + 4H_2O$

Suggested course of oxidations by *Sp. serpens*.

TABLE 4⁴

	Oxygen taken up		CO ₂ given off		Respiratory Quot.	
	Calculated	Observed	Calculated	Observed	Calculated	Observed
1. Acetic acid . . .	1.00	1.03	1.00	1.07	1.00	1.05
2. Propionic acid . . .	3.00	3.00	2.50	0.83	0.83	0.84
3. Lactic acid . . .	2.00	1.98	2.00	1.00	1.00	0.99
4. Pyruvic acid . . .	1.50	1.42	2.00	1.33	1.33	1.28
5. Succinic acid . . .	2.50	2.82	3.00	1.20	1.20	1.15

The oxygen taken up during the oxidation of various substrates by *Sp. serpens*. The calculated values agree with reactions 1-5 postulated in Table 3.

ANAEROBIC RESPIRATION

Reversal of dehydrogenase systems

Before discussing the reactions by which bacteria obtain energy anaerobically the reactions between different dehydrogenase systems must be considered in greater detail.

It was first shown by Quastel and Whetham⁵ that the succinic acid system in the presence of methylene blue could be reversed that is: succinic acid + *MB* \rightleftharpoons fumaric acid + *LMB*. This can be demonstrated most easily by using a Thunberg tube with a hollow stopper. In the tube are placed 1 ml. buffer pH 7.2; 1 ml. $M/500$ succinate; 1 ml. $1/5000$ *MB*; 1 ml. cell suspension; in the hollow stopper 1 ml. $M/50$ fumarate. The whole is evacuated

¹ Clifton, 1946.

⁴ Ibid.

² Barker, 1935.

³ Giesberger, 1936, pp. 98 and 99.

⁵ Quastel & Whetham, 1924.

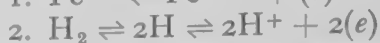
and incubated till the *MB* is reduced; the fumarate is then tipped into the tube which is replaced in the bath; the *LMB* gradually becomes reoxidised. The fact that the reversal is due to enzymic reaction can be shown either by heating the contents of the tube in a boiling-water bath before tipping or by adding a sufficiently powerful antiseptic with the fumarate; in these cases no reoxidation is observed. A precisely similar experiment can be done with the lactic dehydrogenase preparation described above, using lactate, pyruvate and cresyl violet. Even more striking is the case of hydrogenase of *Bact. coli* which catalyses the change of molecular to active hydrogen thus: $\text{H}_2 \rightleftharpoons 2\text{H}$. The fact that this reaction is reversible can be demonstrated by placing 1 ml. buffer and 1 ml. of indicator (in this case methyl viologen) in a series of vacuum tubes, each of which is filled with hydrogen at a different partial pressure. After allowing the reactants to come to equilibrium at the required temperature the degree of reduction of the dye can be estimated by matching the colour against tubes of standard dilutions. It is then found that whilst the *pH* is constant the percentage reduction of the dye is a function of the partial pressure of the hydrogen, i.e. that the system is completely reversible. Moreover, the action of the enzyme is exactly replaced by colloidal palladium as catalyst, the degree of reduction of the dye at any partial pressure of hydrogen being identical in the two cases, provided that all other conditions (temperature and *pH*) are constant.¹

Oxidation-reduction potential

The conception of reduction intensity as a measurable quantity has become useful in biology and is inherent in the idea of reversible dehydrogenase systems. The mathematical theory of this conception has been fully treated in a number of monographs and reviews^{2, 3} and will not be dealt with here; the phenomena upon which the conception rests will be briefly considered. *A* represents a solution of an acid *N* in respect of hydrogen ions in equilibrium with hydrogen gas at 1 atm., *B* is a solution of an iron salt 50% Fe^{+++} and 50% Fe^{++} . *A* and *B* are connected by an agar bridge. *A* is brought into effective contact with H_2 by a platinum electrode *C* covered with palladium. *D* is an electrode dipping into *B*; this catalyses the reaction $\text{H}_2 \rightleftharpoons 2\text{H}$. When *A* and *B* are joined by the conducting bridge (agar saturated with potassium chloride) and *C* and *D* by a wire, a galvanometer (*G*) put into the circuit shows that a current flows in the direction *D*—*C*. If the system is left with the circuit complete the solution in *B* becomes reduced, i.e. the proportion of ferrous salt increases

¹ Green & Stickland, 1934. ² Wurmser, 1930. ³ Mansfield Clark, 1928.

and H^+ ions in A increase. The changes are represented in equilibria 1 and 2:



The flow of the current in the direction $D-C$ indicates the flow of electrons in the opposite direction and the chemical effect of this flow is shown by the reduction of ferric to ferrous salt in B and the oxidation of H_2 to H^+ in A . If, when the vessels are in

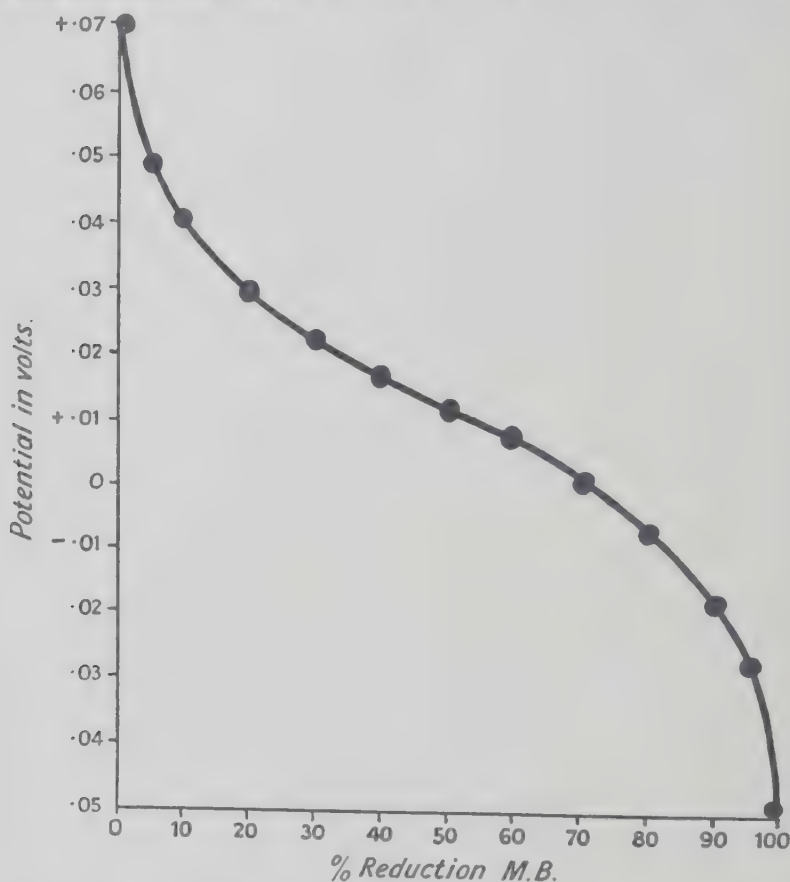


FIG. 3

their original condition (i.e. A N with respect to H^+ in contact with H_2 at 1 atm. and B with 50% of the iron reduced), a potentiometer is introduced in the circuit, a measure of the pressure of the electrons can be made. If we regard the potential of the hydrogen half-cell in the defined conditions as 0, the Eh of the ferrous ferric system is +0.75 v. If the ratio of ferrous to ferric ions be increased to 9 the potential becomes more negative, 0.690 v; if the ratio be altered in the opposite direction ($\frac{1}{9}$) the potential becomes more positive. If Eh is plotted against percentage reduc-

tion an S-shaped curve is obtained similar to that in Fig. 3. E_o is the potential characteristic of the system when the oxidised and reduced forms are equivalent; when this is not the case it can be derived from the equation

$$Eh = E_o - \frac{RT}{nF} \log \frac{Red}{Ox}$$

where Eh is the observed potential, R the gas constant, T the absolute temperature, F the Faraday electrochemical equivalent and n the number of electrons transferred in the reaction formula.

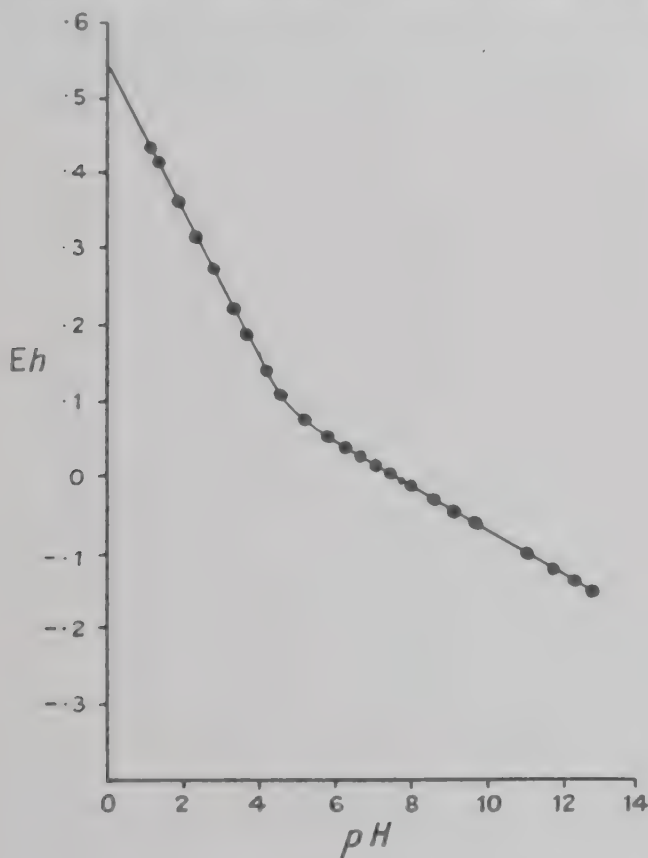


FIG. 4²

The Eh of the system also varies with the pH , a rise in pH value resulting in a more negative Eh (see Fig. 4). The exact change due to pH depends on a number of factors and cannot be detailed here.¹

¹ Mansfield Clark, 1928.

² *Invest. Studies on Oxidation-reduction, No. VIII, Methylene Blue*. U.S. Public Health Reps.

Every *reversible* oxidising-reducing system has a characteristic potential (Eo') when measured against the hydrogen half-cell in the conditions given above and at 30° and $pH\ 7.0$. It will be convenient to consider first the dyes which serve as oxidation-reduction indicators. Fig. 4 represents the change in Eh of the system, methylene blue—leucomethylene blue due to changing ratio of the oxidised and reduced form; $Eo' = Eh$ when $MB/LMB = 1$ in the standard conditions given above. At the point of 50% reduction the Eh is stabilised or buffered and requires a greater relative change in oxidant or reductant to effect a change in Eh than at the ends of the curve. This is analogous to the buffering action of the pH system and has been termed the poisoning action. In order to bring the conception of intensity of oxidation-reduction into line with intensity of acidity as measured by pH , Clark represented it by the symbol rH ; thus, whilst pH represents the negative logarithm of the hydrogen-ion concentration, rH stands for the negative logarithm of the hypothetical hydrogen pressure in equilibrium with the redox system in question:

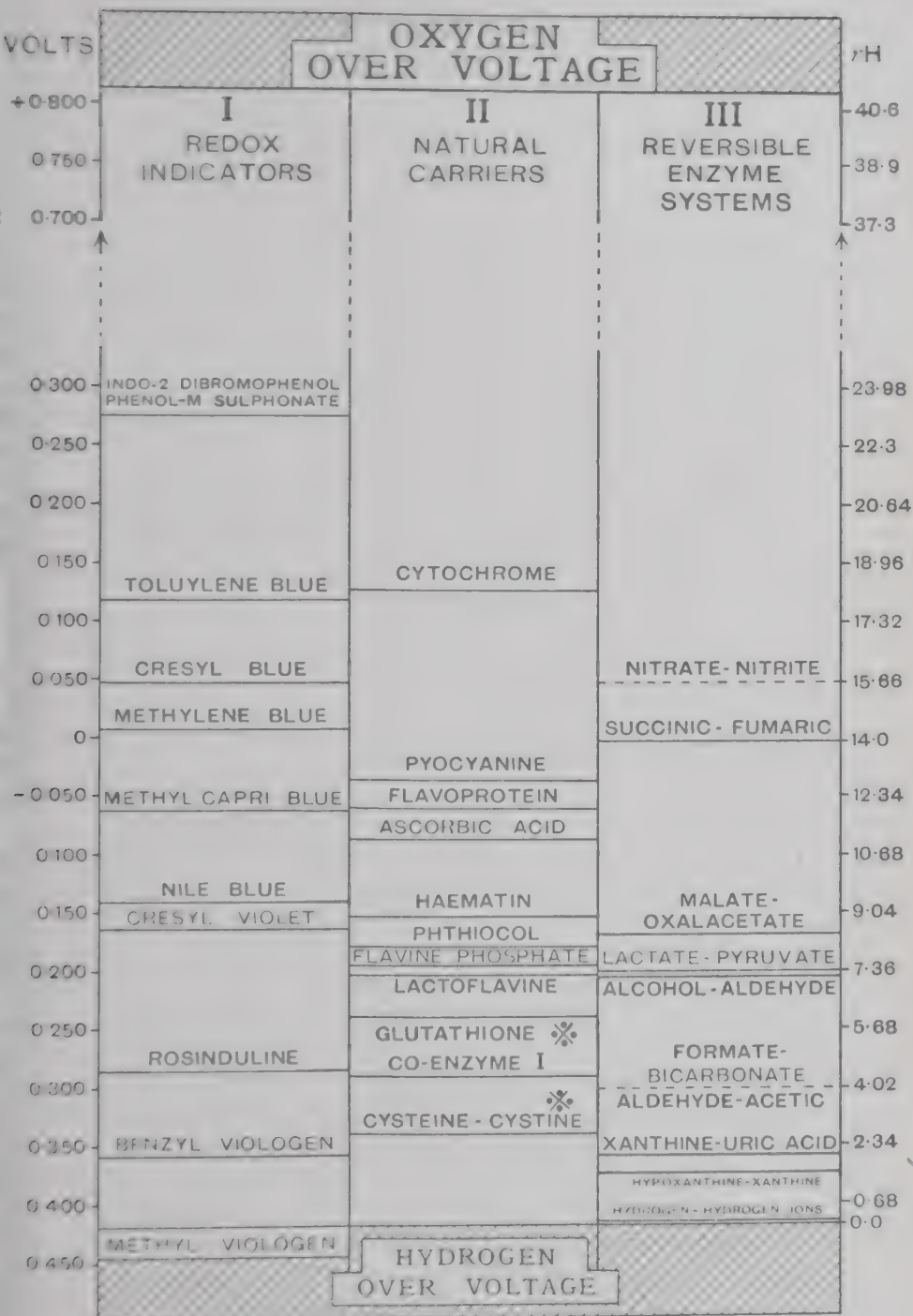
$$rH = 2\left(\frac{F}{RT} \cdot Eh + pH\right)$$

The scale is so arranged that the two points are fixed by the hypothetical oxygen electrode in equilibrium with 1 atm. of oxygen and the pure hydrogen electrode under 1 atm. of hydrogen, the difference in potential between these being 1.23 volts.

The position of any system on the scale determines its behaviour towards all other systems; thus the reduced form of any indicator will reduce the oxidised form of all indicators more positive to it, whilst the oxidised form will oxidise the reduced form of all systems more negative (see Fig. 5).

In the case of reversible redox enzyme systems their behaviour towards the indicators on the scale gives useful information as to the potential at which the oxidant and reductant are in equilibrium. Thus in the case of the lactic dehydrogenase of *Bact. coli* lactate and enzyme will reduce the oxidised form of all indicators whose Eo' at $pH\ 7.0$ is more positive than -0.20 v. (e.g. cresyl violet), whilst the same enzyme in the presence of pyruvate oxidises the reduced form of all indicators more negative than rosinduline but none more positive; * this indicates that the potential of an equimolecular mixture of pyruvate and lactate in the presence of the enzyme must lie somewhere between $Eh\ -0.167$ and -0.281 . This can be shown experimentally as follows:

* Beyond these limits the reaction, if it occurs, is at an immeasurably slow rate.



E_0 at pH 7.0 and 30°

* THE REVERSIBILITY OF THIS SYSTEM IS DOUBTFUL

FIG. 5

In Fig. 6 *A* is the reaction vessel connected to a vacuum pump ; in this is immersed a gold or platinum electrode connected to a potentiometer. *A* is drawn out to a capillary which is filled with agar saturated with KCl and dipping into a saturated solution of KCl. This is connected by bridges of agar saturated with KCl

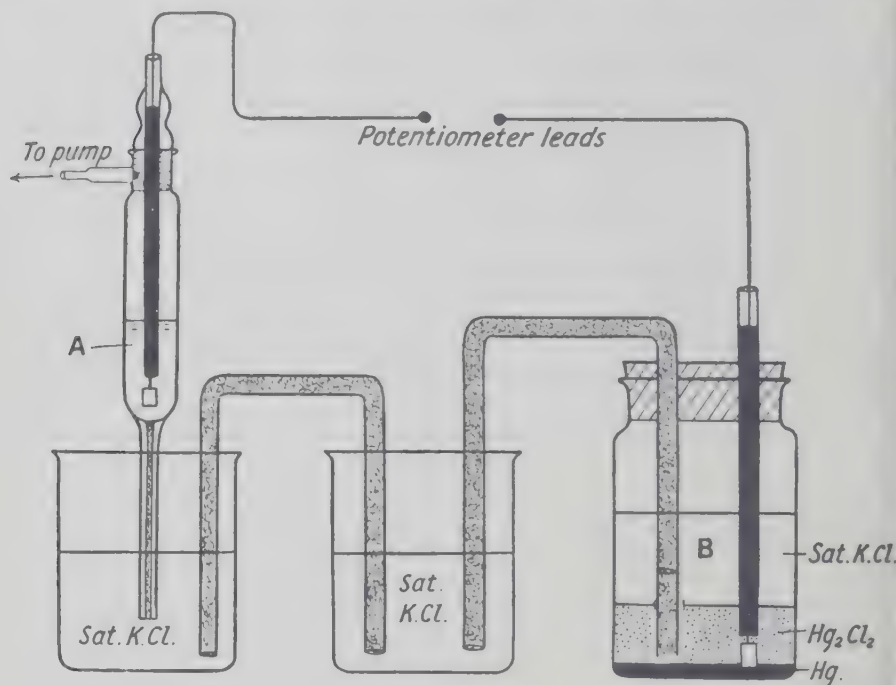


FIG. 6

with a calomel half-cell carrying the second electrode. *A* contains buffer at pH 7.0, enzyme preparation (lactic dehydrogenase) and lactate and pyruvate in varying proportions and a trace of cresyl violet. The vessel is evacuated and the potentiometer reading recorded ; if the *Eh* is plotted against the ratio lactate/pyruvate a curve as seen in Fig. 7 results.

In a similar way the position on the potential scale of succinate and fumarate and other reversible systems is determined. Fig. 5, I, shows the *Eo'* at pH 7.0 of some of the redox indicators ; II that of some naturally occurring carrier substances ; III that of some reversible enzyme systems. We will now consider what information can be gained from this method of regarding the subject. In the first place, the oxidised form of any of the substances in column I will rapidly oxidise the reduced form of any other substance lying below it in the scale, but any substance lying above it is not oxidised at all or only at a low rate ; conversely, the reduced form of any substance will reduce the oxidised form

of any other substance lying above it, but any substance below it is either not reduced at all or only at a low rate. Such oxidations or reductions occur without the intervention of any enzyme or catalyst though at very differing rates. On the other hand, supposing the reduced form of hypoxanthine be put in contact with

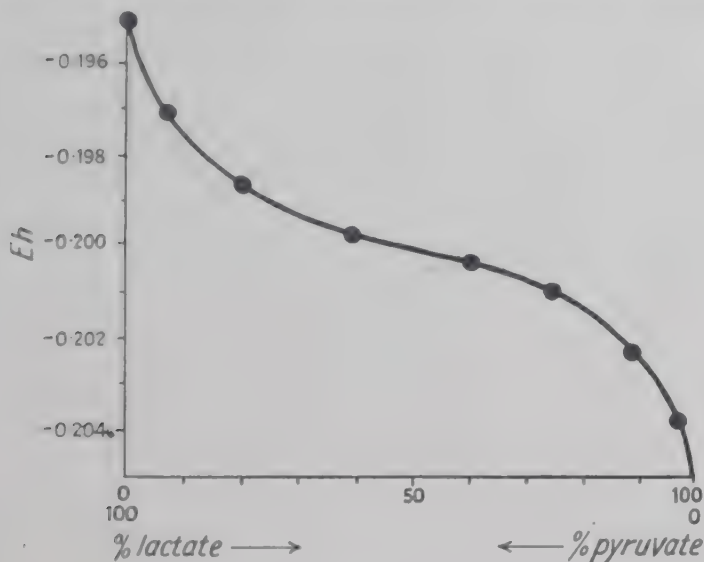


FIG. 7

the oxidised form of methylene blue, no action occurs except in the presence of the appropriate enzyme (xanthine oxidase) to catalyse the transfer of hydrogen to the dyestuff. In the presence of its dehydrogenase the oxidised form of any substance in column III will oxidise the reduced form of any substance in column I lying below it, whilst the reduced form of any substance in III will reduce the oxidised form of any substance in column I lying above it, but none of those lying below it except at a very low rate. When we consider the relationship of the substances in column III among themselves a different state of affairs is manifest. On the analogy of what occurs between substances of column I, one would expect that hypoxanthine would reduce fumarate, provided that both enzymes were present, xanthine oxidase to oxidise the donator, hypoxanthine, and succinic dehydrogenase to activate the acceptor, fumarate. Actually in these circumstances no reaction occurs until one of the redox dyes, say cresyl violet, is added; fumarate is then reduced to succinate by hypoxanthine, but no reaction occurs between succinic acid and xanthine; in fact, the hypoxanthine reduces the cresyl violet, which in turn reduces the fumarate.

Hypoxanthine + cresyl violet $\xrightarrow[\text{oxidase}]{\text{xanthine}}$ reduced cresyl violet + xanthine

Fumarate + reduced cresyl violet $\xrightarrow[\text{dehydrogenase}]{\text{succinic}}$ succinate + cresyl violet

In order for the carrier to operate efficiently its *Eo'* should lie between those of the principal reactants.

Intracellular anaerobic oxidoreductions

When cell suspensions are used, oxidoreductions, such as that described in the preceding paragraph, can be shown to occur without the addition of a carrier ; for example, molecular hydrogen reduces both fumarate and nitrate in presence of *Bact. coli*. If instead of bacterial cells the isolated dehydrogenase systems are used it is found that the components of the systems then fail to interact ;¹ if, however, an indicator, which need be present only in traces, be added to the system the component of the most positive system is reduced. This is clearly seen from Table 5.

TABLE 5²

<i>Negative system</i>		<i>Indicator</i>	<i>Positive system</i>	
<i>Enzyme</i>	<i>Substrate</i>		<i>Enzyme</i>	<i>Substrate</i>
Formic dehydrogenase (<i>Bact. coli</i>)	Formate	Benzyl viologen	Nitratase	Nitrate
Lactic dehydrogenase (<i>Bact. coli</i>)	Lactate	Ethyl Capri blue	Nitratase (<i>Bact. coli</i>)	Nitrate
Xanthine oxidase (milk)	Hypoxanthine	Methyl viologen	Succinoxidase (heart muscle)	Fumarate
Hexosemonophosphate dehydrogenase (yeast)	Hexosemonophosphate	Ethyl Capri blue	Succinoxidase (heart muscle)	Fumarate
Glucose dehydrogenase (liver)	Glucose	Ethyl Capri blue	Succinoxidase (heart muscle)	Fumarate
Xanthine oxidase (liver)	Hypoxanthine	Ethyl Capri blue	Succinoxidase (liver)	Fumarate
Lactic dehydrogenase (liver)	Lactate	Ethyl Capri blue	Succinoxidase (liver)	Fumarate
Xanthine oxidase (milk)	Hypoxanthine	Methyl viologen	Lactic dehydrogenase (<i>Bact. coli</i>)	Pyruvate

If now such systems interact in the cell one must postulate some carrier playing the part of the indicator ; coenzyme I plays this part.³

Thus

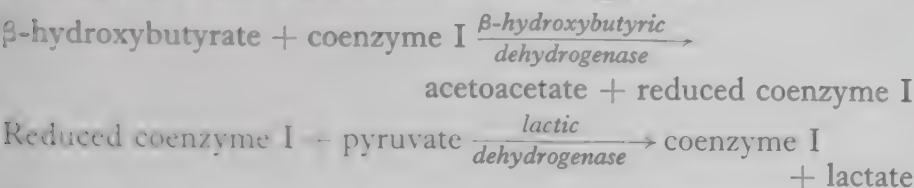
β -hydroxybutyrate + β -hydroxybutyric-dehydrogenase + pyru-

¹ Green, Stickland & Tarr, 1934.

² Ibid., *Biochem. J.*, 28, 1815.

³ Dewan & Green, 1937.

pyruvate + lactic dehydrogenase give no reaction, but in the presence of coenzyme I and diaphorase the following occurs:



Similarly the malic dehydrogenase extracted from *Bact. coli* requires the addition of coenzyme I (and diaphorase) in order to oxidise *L*-malate to oxaloacetate.¹

The electrode potential measurements are frequently used to measure the reducing level of culture media. It is not now claimed that this gives a measure of the reduction potential of the organism since this obviously has no meaning, nor that it measures the reducing level of the cell surface, for no systems functioning there can affect the electrode. When steady readings are in fact given by the electrode this can be shown to be due to substances liberated from the medium by the action of the cells.

Thus it has been shown that electrodes plunged in anaerobic bacterial suspensions in *M*/20 buffer at *pH* 7.0 undergo a slow fall; this level is unaltered by the addition of succinate, but formate or glucose causes a rapid drop (Fig. 8). The result is approximately the same if formate is added in place of glucose and vice versa.

This final level was approximately the same when the electrodes were protected from contact with the bacteria by a collodion sac,

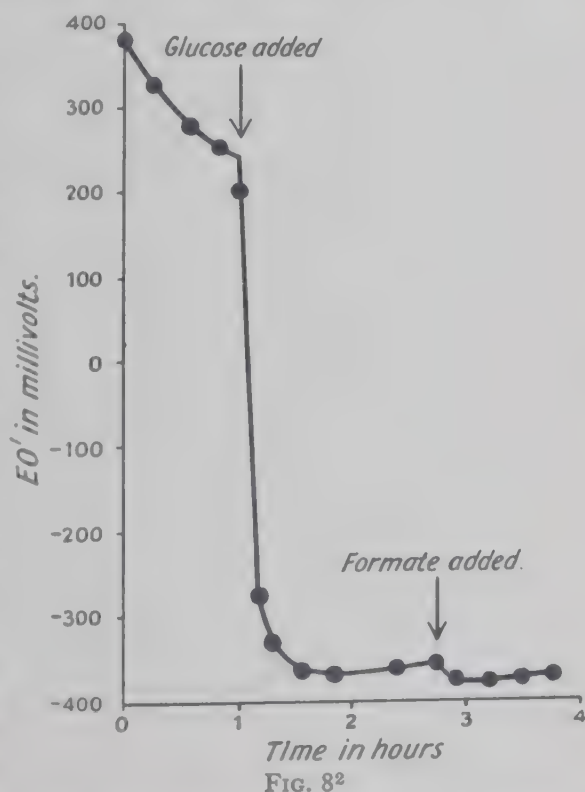


FIG. 8²

¹ Gale & Stephenson, 1939.

² Yudkin, *Biochem. J.*, **29**, 1132, 1935.

showing that the potential level is not due to reactions occurring on the bacterial surface but to diffusible substances liberated by bacterial action.¹

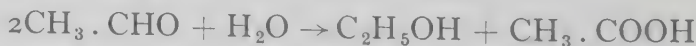
The fact that certain growth phenomena, e.g. the germination of the spores of *Cl. tetanum*,² can occur only in conditions which give certain potential readings with the electrode shows that the presence of substances associated with (not necessarily causing) these readings conditions the growth phenomena in question.

Oxidoreductions

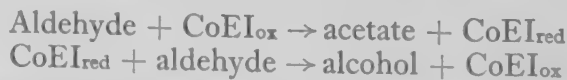
It has been shown earlier that oxidations in which O_2 is finally involved proceed by a series of dehydrogenations in which $2H$ is successively transported from one molecule to another. At each of these stages energy is liberated and can be used by the cell for synthetic purposes. Thus anaerobes and facultative anaerobes living anaerobically use, in many cases, the same oxidative mechanisms as aerobes with the exception of those concerned directly with the final transfer of $2H$ to O_2 , viz. the cytochromes and cytochrome oxidase, peroxidase, and the direct oxidative enzymes.

Chief among the substances providing energy for anaerobic life are the carbohydrates and it must be noted that all types of fermentation are exergonic. For this reason sugars will often supply the sole source of carbon and energy for the life of fermenting organisms; even where a fermenting anaerobe can grow on a carbohydrate-free medium such as peptone the addition of glucose greatly increases the growth. The anaerobic breakdown of carbohydrates involves a complicated series of interactions including oxidoreductions, dismutations, phosphorylations and special carrying mechanisms. This subject is so large that it is treated in a separate chapter for convenience rather than from any logical demarcation of the subject.

When oxidoreductions occur between two similar molecules the reaction is known as a dismutation. Thus:



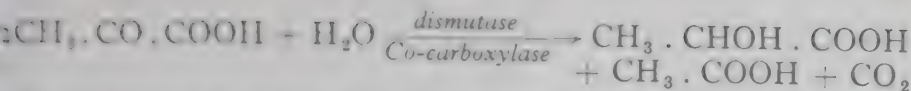
Actually coenzyme I participates in this reaction which is more precisely written thus:



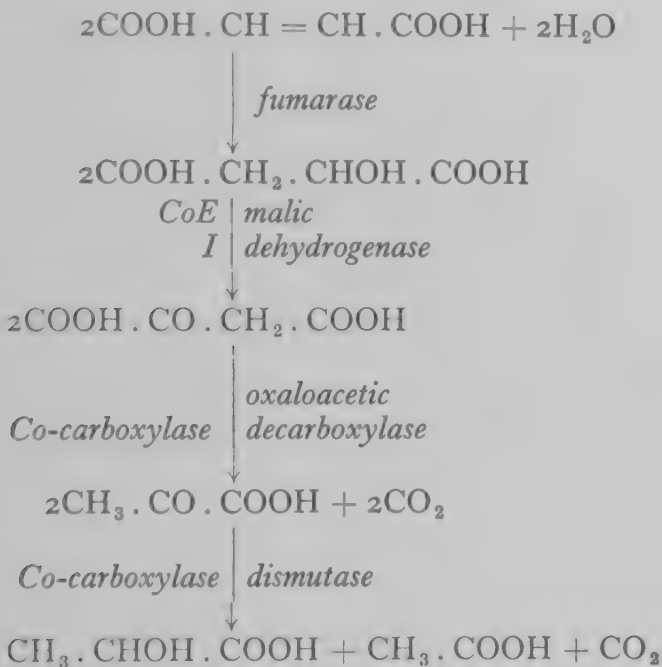
Dismutations may involve the rupture of one of the reacting molecules by some non-oxidative process such as hydrolysis; the dismutation of pyruvic acid by the gonococcus (*N. gonorrhæa*) is an example of this.

¹ Yudkin, 1935.

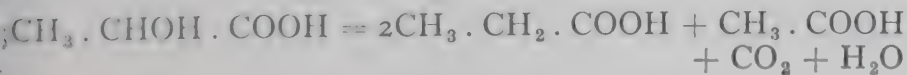
² Fildes, 1929.



The oxidation of a substrate to its final end products often involves the production of intermediate products by non-oxidative enzymes such as deaminases, hydrolytic enzymes and carboxylases. The oxidative breakdown of fumaric acid illustrates this.



Molecules which cannot undergo further anaerobic decomposition become the end products of anaerobic metabolism; such in the case of *Esch. coli* and most members of that group are succinate, acetate and lactate. With propionibacteria, on the other hand, acetate can undergo a further dismutation:



Anaerobic mechanisms for obtaining energy

The majority of bacteria are either facultative or obligatory anaerobes and the main problem confronting these organisms is that of obtaining energy for cell synthesis without employing molecular oxygen. It is important to notice that the amount of energy available as a result of oxidoreductions is less than when oxygen is the hydrogen acceptor. In many cases the ΔF of the reaction is not known but the ΔH indicates that this is the case.

1. $\text{CH}_3 \cdot \text{CHOH} \cdot \text{COOH} + \frac{1}{2} \text{O}_2$
 $= \text{CH}_3 \cdot \text{CO} \cdot \text{COOH} + \text{H}_2\text{O} + 51.9 \text{ K. cal.}$
2. $\text{CH}_3 \cdot \text{CHOH} \cdot \text{COOH} + \text{KNO}_3$
 $= \text{CH}_3 \cdot \text{CO} \cdot \text{COOH} + \text{H}_2\text{O} + \text{KNO}_2 + 30.7 \text{ K. cal.}$
3. $\text{CH}_3 \cdot \text{CHOH} \cdot \text{COOH} + \text{COOH} \cdot \text{CH} : \text{CH} \cdot \text{COOH}$
 $= \text{CH}_3 \cdot \text{CO} \cdot \text{COOH} + \text{COOH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH} + 16 \text{ K. cal.}$

Respiratory mechanisms of the Clostridia

This group of strict anaerobes is characterised by (1) its low tolerance for oxygen ; (2) the formation of spores ; (3) the absence (in all the members tested) of all mechanisms connected with molecular oxygen, viz. cytochrome and cytochrome oxidase ; peroxidase and catalase ; (4) its elaborate food requirements. Those members of the group which are vigorous fermenters doubtless rely on this as a source of energy, but those which develop on protein or protein digests alone require special examination. It might be supposed that since many facultative anaerobes also develop anaerobically on protein digests, no special mechanism in the case of the *Clostridia* need be sought for ; special examination, however, shows that this is not the case. Suspensions of *Cl. sporogenes*, for example, do not possess a number of dehydrogenases characteristic of *Esch. coli* and facultative anaerobes in general, but vigorously dehydrogenate certain amino-acids. Furthermore the principal substances known to act as hydrogen acceptors with facultative anaerobes are inactive with this organism. Several amino-acids are, however, vigorous hydrogen donators, whilst others can be shown to act as acceptors (see Tables 6 and 7).

TABLE 6¹

SUBSTANCES ACTING AS HYDROGEN DONATORS WITH *Cl. sporogenes*
 Velocity of oxidation of alanine = 100

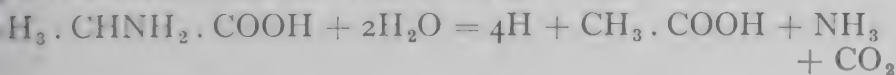
Substrate	Rate of oxidation	Substrate	Rate of oxidation
Sodium formate . .	0	<i>l</i> -Aspartic acid . .	5
„ acetate . .	0	<i>l</i> -Glutamic acid . .	2
„ propionate . .	0	<i>l</i> -Arginine . .	0
„ lactate . .	0	<i>l</i> -Lysine . .	0
„ succinate . .	0	<i>l</i> -Histidine . .	<2
„ pyruvate . .	40	<i>l</i> -Proline . .	0
Glucose . .	0	<i>l</i> -Hydroxyproline . .	0
Glycine . .	0	<i>dl</i> -Serine . .	0
<i>l</i> -Alanine . .	100	<i>l</i> -Tyrosine . .	<2
<i>d</i> -Alanine . .	0	<i>l</i> -Tryptophan . .	<2
<i>l</i> -Valine . .	60	<i>l</i> -Leucine . .	100
<i>l</i> -Phenylalanine . .	10		

¹ Stickland, *Biochem. J.*, 28, 1748.

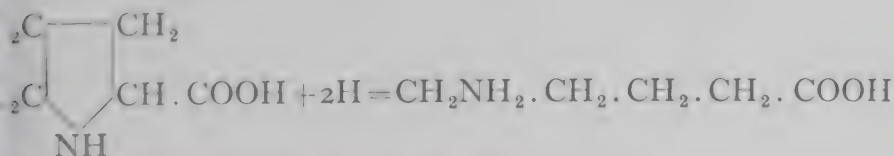
TABLE 7¹SUBSTANCES ACTING AS HYDROGEN ACCEPTORS WITH *Cl. sporogenes*

Substance	Indicator	Reoxidation
Sodium nitrate .	Benzyl viologen	o
" fumarate	" "	o
<i>dl</i> -Serine . .	" "	o
<i>l</i> -Aspartic . .	" "	o
<i>l</i> -Glutamic . .	" "	o
<i>l</i> -Arginine . .	" "	o
<i>l</i> -Lysine . .	Neutral red	o
<i>l</i> -Histidine . .	Benzyl viologen	o
<i>l</i> -Tyrosine . .	" "	o
<i>l</i> -Proline . .	" "	++ ++
" . .	Neutral red	++ ++
" . .	Rosinduline	++ ++
" . .	Phenosafranine	++ ++
" . .	Ethyl Capri blue	—
<i>l</i> -Hydroxyproline	Benzyl viologen	++ ++
"	Neutral red	++ ++
Glycine . .	Methyl viologen	++ ++
" . .	Benzyl viologen	+
" . .	Phenosafranine	—

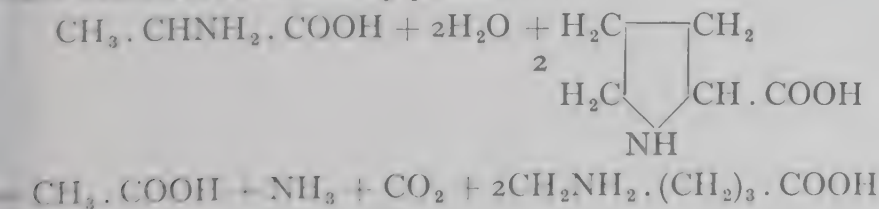
When a suspension of the organism acts on a donator and acceptor simultaneously double decomposition occurs, involving deamination and oxidation of the donator and reduction and deamination of the acceptor. Quantitative estimation of the products resulting from these reactions shows that they occur as follows:



Reduction of proline²



Successive oxidation of alanine by proline:



Ornithine when acting as hydrogen acceptor is deaminated and reduced, forming δ -aminovaleric acid; glycine gives ammonia and acetic acid; the products resulting from the oxidation of

¹ Suckland, *Biochem. J.*, **28**, 1752.

² *Ibid.* 1934, 1935 (1), (2).

cysteine and the reduction of arginine are at present uncertain. The energy available from this series of oxidoreductions is difficult to compute; the fact that the reactions occur at a rate comparable with that of aerobic oxidations (Q_{O_2} alanine + proline = 50) makes it probable that these reactions represent the respiratory mechanisms of the cell.^{1, 2}

The peculiar reaction between organisms of the *Clostridia* and atmospheric oxygen is still rather obscure. The discovery of a class of microbes unable to develop in the presence of free oxygen was made by Pasteur,³ who recognised this peculiar property in the butyric fermenter (*Cl. butyricum*), "êtres qui vivent sans gaz oxygène et que l'air fait périr." This unique reaction to oxygen differentiates this class of organisms not only from all other bacteria but from all other living forms. The problem of how organisms possessing this intolerance for oxygen can find natural conditions in which to develop at once presented itself to Pasteur, who rightly assigned to the aerobes, with which anaerobes are constantly associated in nature, the role of rendering their natural habitat anaerobic. This opinion was fully accepted, and led to the widespread adoption of a system of cultivating anaerobes "aerobically" in mixed culture with aerobes.^{4, 5, 6, 7} Subsequently certain investigators came to doubt the sufficiency of a simple respiratory function to account for the action of the associated aerobes, mainly in view of the fact that even on the surface of an agar slant anaerobes are able to develop if aerobes are also present. Kedrowski,⁸ for example, postulated the existence of an enzyme secreted by aerobes which enables anaerobes to develop aerobically. This view was temporarily confirmed by the observation that even after chloroform treatment aerobes retain the power to enable anaerobes to grow "aerobically."⁹ Novy,¹⁰ however, finally disposed of this theory by showing that aerobes can bring about the growth of anaerobes even when grown apart from them in a separate limb of a closed H tube. In such circumstances the aerobe develops first, completely removing the oxygen from the air space and hence from the culture media; the development of the anaerobe in the other limb of the H tube then follows. Not only aerobes can function in this way, but also actively respiring tissue such as fresh sterile potato, etc., and, indeed, it is to such anaerobiosis that the development of tetanus and other anaerobic infections in deep wounds is largely due.

The sensitivity of the *Clostridia* to oxygen still awaits satisfactory explanation. The view has been advanced that, in the absence

¹ Stickland, 1934.

² Woods, 1936 (2).

³ Pasteur, 1861 (2), 1863.

⁴ Ibid.

⁵ Roux, 1887.

⁶ Penzo, 1891.

⁷ Novy, 1925.

⁸ Kedrowski, 1895.

⁹ Ibid.

¹⁰ Novy, 1925.

of catalase, aerobic oxidation occurs, producing sufficient H_2O_2 to retard development and even to kill vegetative forms. There are, however, objections to this view. Prolonged aeration on media containing material oxidisable by these organisms fails to produce H_2O_2 in demonstrable amounts^{1, 2} and added catalase does not result in aerobic growth; furthermore, washed suspensions of *Cl. sporogenes* aerated in presence of broth or oxidisable substrate fail to take up oxygen. In all these respects the *Clostridia* differ from facultative anaerobes which have no catalase and whose aerobic development is retarded by H_2O_2 . On the other hand, M'Leod and Gordon have obtained indirect evidence of peroxide production by the use of deep tubes of agar containing heated blood ("chocolate agar"). At a level of a few millimetres from the surface where the penetrating oxygen met the bacterial growth a green band was formed attributed to the oxidising effect of H_2O_2 on hæmoglobin. It is possible that H_2O_2 may arise from secondary chemical processes occurring in the medium apart from any direct process of oxidation connected with the cell. For instance, as a result of the action of some anaerobes on broth—SII compounds are produced which may, on contact with oxygen, give H_2O_2 . More detailed work by Hart and Anderson has, however, shown that the green coloration produced in "chocolate" agar can be reproduced anaerobically by the action of reducing systems on laked blood and also from oxy-, meth- and carboxyhæmoglobin. The process appears to involve a reduction of oxyhæmoglobin to reduced hæmoglobin followed by an oxidation. Reducing systems producing the pigment include ascorbic acid, cysteine, bacterial suspensions with hydrogen donors; the identity of the green pigment is uncertain but it resembles "green hæmin." Thus there seems no reason to regard the green zone produced by anaerobes in "chocolate" agar as due to hydrogen peroxide.³

The oxidoreductions so far considered have involved two organic substrates. Bacteria are, however, able to use their dehydrogenase systems to reduce certain inorganic molecules as hydrogen acceptors; this involves the activation of the acceptor in each case by a special enzyme.

The reduction of nitrate

The reduction of NO_3^- to NO_2^- has long been used as a diagnostic test for certain groups of bacteria. The $2H$ is transferred to the NO_3^- by the dehydrogenases and the appropriate coenzyme, the reaction requiring in addition the enzyme nitratase. This enzyme has so far only been studied in the intracellular state.⁴

¹ Callow, 1923.

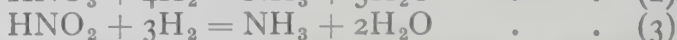
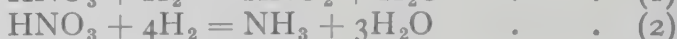
² Hart & Anderson, 1933 (1), (2).

³ M'Leod & Gordon, 1925.

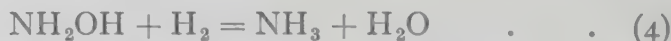
⁴ Stickland, 1931.

Using lactate as the hydrogen donator the system consists of lactate, lactic dehydrogenase, coenzyme I, nitrate and nitratase. In order to annul the effect of other enzymes which might interfere, toluene-treated cells are used. Oxidation by nitrate occurs at about one-tenth the rate of oxidation by O_2 . Both systems are inhibited by cyanide, 50% inhibition of nitratase occurring at $0.0001 M$ and that of the oxygen system at $0.001 M$ cyanide respectively. CO causes no inhibition. The system is not reversible. For cells possessing nitratase, therefore, NO_3^- can replace O_2 quantitatively, but it is less efficient for several reasons: (1) the optimal rate of oxidation is less; (2) the energy delivered per mol. of substrate oxidised is lower (see p. 46) and the nitrite formed has a bacteriostatic action when a concentration of about $0.003 M$ is reached.

Many organisms reducing nitrate can, under appropriate conditions, reduce the NO_2^- formed to NH_3 , with hydroxylamine as an intermediate product. With organisms possessing hydrogenase this can be shown manometrically;¹ it was first demonstrated for *Cl. welchii* but many strains of *Esch. coli* have been used, and the following reactions shown to occur:



The intermediate formation of hydroxylamine is strongly suggested by the above reactions and hydroxylamine itself can be reduced to NH_3



though it has not so far been isolated as an intermediate. A qualitative test for it has, however, been obtained on the completion of nitrite reduction by several species.² The relative rates of reactions (1) to (4) vary with different strains; usually reaction (1) is the fastest, which accounts for the accumulation of nitrite. It should be noted, however, in applying nitrate reduction as a diagnostic test that, given appropriate conditions and a sufficient length of time, nitrate may be reduced without any accumulation of nitrite.

The reduction of sulphate

Whilst the reduction of nitrate is brought about by many facultative anaerobes, the reduction of sulphate is much less common. It was first shown by Beijerinck³ that the hydrogen sulphide produced in mud arises anaerobically by the reduction of sulphates; van Delden⁴ first obtained pure cultures of the organisms con-

¹ Woods, 1938.

³ Beijerinck, 1895.

² Lindsey & Rhines, 1932.

⁴ van Delden, 1904.

cerned (*V. desulphuricans* and *V. aestuarii*) in a medium in which sulphate was reduced to sulphide anaerobically at the expense of lactate and malate, the energy for development being derived from the reduction of the sulphate; Elion¹ isolated a thermophilic organism of the same type. All these organisms are strictly anaerobic. They form spores in natural conditions but not on laboratory media, except when cultivated at 45° or over.² They may be isolated by the delightful method of van Delden, which consists in inoculating soil or river mud into ordinary media containing 0.5% sodium sulphate, incubating anaerobically and then plating on broth agar containing sulphate and iron salts (0.005% potassium iron sulphate); after anaerobic incubation the colonies responsible for production of sulphide are distinguished by being jet black owing to the precipitation of iron sulphide. All members of this group can be grown anaerobically on an inorganic medium consisting of lactate or glucose and sulphate. Baars³ has made an exhaustive study of the group and has shown that the sulphate reduced is quantitatively equivalent to the carbon compound oxidised, and that the species differ only in the carbon compounds which they are able to oxidise.

A member of the group isolated from river mud⁴ is able to reduce sulphate by molecular hydrogen owing to its possessing hydrogenase. By using washed suspensions it was shown that the hydrogen absorbed was equivalent to the sulphate reduced and the hydrogen sulphide formed.

In addition to sulphate other sulphur compounds are reduced by these organisms, viz. sulphite, hyposulphite, thiosulphate and also sulphur.⁵

Sulphate reducers occasionally give rise to extensive corrosion of gas mains. The necessary conditions are anaerobiosis, a clay soil supplying sulphate and sufficient organic matter to provide hydrogen donors for the sulphate reduction; the H₂S liberated in this way has been shown experimentally to cause extensive corrosion of steel^{6, 7} under laboratory conditions.

The reduction of tetrathionate

The inclusion of tetrathionate in laboratory media is used to favour the growth of organisms of the *Salmonella* group. Favourable response to growth on such media has been found to be due to the presence of an enzyme reducing tetrathionate to thiosulphate and involves the use of some substance acting as a hydrogen donor.^{8, 9}

¹ Elion, 1924.

² Starkey, 1938.

³ Baars, 1930.

⁴ Stephenson & Stickland, 1931 (2).

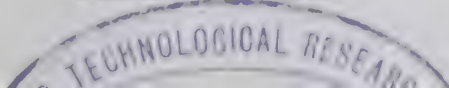
⁵ Baars, 1930.

⁶ Bunker, 1939.

⁷ Starkey & Wight, 1945.

⁸ Knox, Gell & Pollock, 1943.

⁹ Pollock & Knox, 1943.





The adaptive nature of this enzyme (tetrathionase) will be discussed in a later chapter.

The reduction of selenite and phosphate

Selenites are easily reduced by bacteria, both aerobes and facultative and strict anaerobes (the latter only tolerating low concentrations of the salt). The selenite is reduced to metallic selenium, seen on solid media as a red streak along the line of growth;^{1, 2} microscopically the solid particles of selenium can be seen deposited inside the cell. It does not appear that this reduction operates usefully in the cell economy, since such aerobic organisms as *Bac. subtilis* and *Chr. prodigiosum* are not enabled to develop anaerobically by its means. Selenic acid is reduced in the same way as selenious acid,³ and tellurites behave like selenites, tellurium being deposited inside the cell. The concentrations of selenite best adapted for showing this reduction are 1 part in 100,000 of selenious acid for strict anaerobes and 1 part in 25,000 to 1 in 50,000 for aerobes and facultative anaerobes.

The bacterial reduction of phosphate to phosphine has been reported to occur in a number of putrefactions of protein material.⁴ Rudakov⁵ reported the isolation of an organism able to reduce phosphate successively to phosphite, hypophosphite and phosphine.

The reduction of carbon dioxide and the production of methane

The production of methane was early associated with the decomposition of cellulose in mud^{6, 7} and in the intestine of the ox.⁸ Omeliansky⁹ in particular carried out a fermentation of cellulose by a *Clostridium* believed to be in pure culture in which methane and various fatty acids were obtained.

It is now fairly clear that the association of methane production with cellulose fermentation is fortuitous and due to the fact that these processes both occur in the alimentary tract of the ruminant where anaerobic conditions and large quantities of lower fatty acids occur. The origin of methane was first clearly indicated by the important work of Sohngen,¹⁰ who showed that the calcium salts of various fatty acids could be quantitatively decomposed to carbon dioxide and methane. Sohngen was unable to obtain pure cultures of his organisms, but carried out his fermentations with

¹ Klett, 1900.

² Scheurlen, 1900.

³ Levine, 1925.

⁴ Barrenscheen & Beckh-Widmanstetter, 1923.

⁵ Rudakov, 1927.

⁶ Popoff, 1875.

⁷ Hoppe-Seyler, 1886.

⁸ Tappenheimer, 1883, 1884.

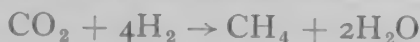
⁹ Omeliansky, 1902, 1904 (1), (2).

¹⁰ Sohngen, 1910.

enrichment cultures obtained by repeated inoculations into media in which the fatty acid in question was the sole source of carbon ; the difficulty of obtaining pure cultures has been experienced by most subsequent workers in the field.¹ Sohngen's most illuminating discovery was that cultures decomposing formate according to the equation



also synthesised methane from carbon dioxide and hydrogen :



Following Sohngen's observation methane was obtained by the strictly anaerobic fermentation of many organic compounds, e.g. methyl, ethyl and butyl alcohols, fatty acids and compounds derived from sewage, all done in impure cultures, and hence, to quote Barker, "there is no certainty that methane arises by a direct conversion of all these organic substrates."

Kluyver in 1936 drew attention to the role of methane as the final and most reduced stage of oxidoreduction and pointed in the direction in which lay the solution of the problem.

In recent years the nature of methane production has been elucidated by the researches of Barker. This worker was the first to succeed in isolating methane bacteria in pure culture. This he did by enrichment cultures, using a very simple medium, II. I, containing 1-2% of some organic compound ; in the case of organic acids the Ca salt was used and in other cases CaCO_3 was added. This provides the CO_2 which acts as the acceptor for 2H . The difficulty of isolating methane organisms is due to the fact that they are all strict anaerobes which do not generally form spores ; in the final stages of the isolation the organisms are protected from O_2 by the incorporation of Na_2S in the medium. These organisms are slow growers, which is doubtless connected with the fact that they depend for their development on oxidoreductions where CO_2 is the acceptor, delivering very little energy.

The technique for the isolation of these organisms has been fully worked out by Barker^{2, 3} and is a logical application of the biological and biochemical characteristics of the species. Barker has isolated organisms which during their development bring about the following reactions which can all be expressed by the general equation

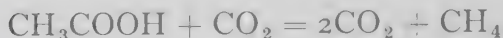
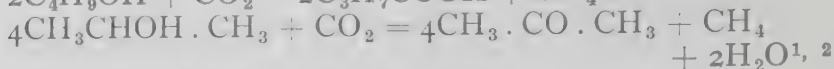
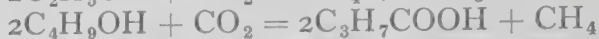
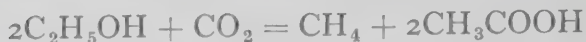
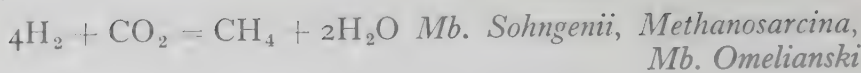


where H_2A represents any compound for which the organism possesses a dehydrogenase.

¹ Barker, 1936 (2).

² Ibid.

³ Ibid., 1940.



The rate of CO_2 absorption in reaction 1 depends on CO_2 concentration and attains half maximal rate at $7 \times 10^{-5} M$. When the partial pressure of CO_2 is kept constant the rate is nearly independent of $p\text{H}$ over the range 5.8–7.4, whilst the rate declines with increasing $p\text{H}$ over the range 6.4–8.6 in which CO_2 tension decreases and bicarbonate tension increases. Hence it appears that CO_2 is the substance reduced and not the HCO_3^- ion.³

The use of radioactive C has provided valuable information as to the origin of the C used by *Mb. Omelianski* in cell synthesis.⁴ Washed cell suspensions were incubated with radioactive CO_2 and ethanol. At the end of the incubation period carbonate prepared by the combustion of (1) the cells, (2) the CH_4 , (3) the acetic acid was examined for radioactivity; this was found in (1) and (2) but not in (3), showing that CO_2 is reduced to CH_4 and cell material, the latter forming about 1.5% of the total C reduced. A similar result was obtained with *Methanosarcina*. This is important as indicating that the organism uses CO_2 for synthesis of cell material.

A case previously studied⁵ is a less clearcut example of simple CO_2 reduction. An organism derived in pure culture from river mud contained hydrogenase and was found to reduce by this means a series of 1-carbon compounds to CH_4 . No compounds with more than one carbon atom were attacked. The following six reactions were brought about quantitatively by washed suspensions of the organism:



(1), (2) and (3) proceed quickly, (4), (5) and (6) comparatively slowly, and evidence was obtained that formic acid was first decomposed by formic hydrogenlyase



¹ Barker, 1936 (1), (2).

² Ibid., 1941.

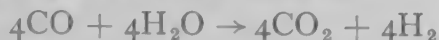
³ Ibid., 1943.

⁴ Barker, Ruben & Kamen, 1940.

⁵ Stephenson & Stickland, 1933 (2).

and CH_4 then synthesised by reaction (2). With regard to (5) and (6) a preliminary reduction to $\text{H} \cdot \text{COOH}$ may occur, giving CH_4 by reactions (1) and (2).

With regard to reaction 4 Schnellen¹ has shown that two pure cultures of methane organisms, *Ms. Barkerii* and *Mb. formicum* convert CO into CH_4 in the absence of H_2 as well as in its presence. In the absence of H_2 , CO reacts with water



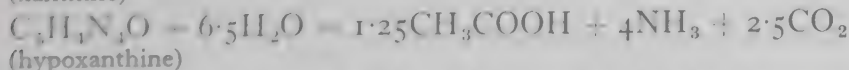
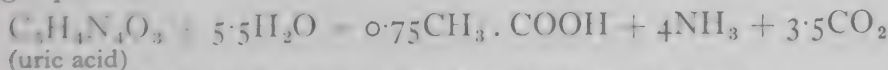
the CO_2 and H_2 subsequently reacting according to reaction 2.

The reduction of CO_2 to acetic acid

Analogous to the reduction of CO_2 to CH_4 is its reduction to acetic acid. This was first explored by Wieringa,² who isolated from canal mud a *Clostridium* which reduced CO_2 to acetic by molecular hydrogen



offering an exact analogy to the Sohngen reaction. This same reduction process, using different hydrogen donators, has now turned up in several groups. One suitably considered here is an interesting group of *Clostridia* isolated from the soil, dependent on uric acid or its reduction products, xanthine, hypoxanthine and guanine for its energy and growth requirements. The specificity of this organism (*Cl. acidi-urici*) is very high, no other compounds among a large number tested being attacked, with the exception of glycine, which is reduced to acetic acid in the presence of uric acid. The four purines mentioned all serve as energy source and supply the growth requirements of the organism in strictly anaerobic conditions, acetic acid and CO_2 being the only products formed; the decomposition occurs in accordance with the following equations:



When uric acid was attacked by this organism in the presence of radioactive CO_2 the C^* was found in both C atoms of the acetic acid. This constitutes strong evidence that CO_2 is reduced to acetic in the course of the fermentation of the purines, the CO_2 finally appearing as end product in the above equations, being in excess of that required to act as hydrogen acceptor.^{3, 4} This

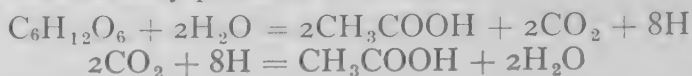
¹ Schnellen, 1947.

² Barker & Beck, 1941 (1), (2).

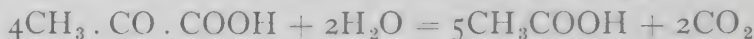
³ Wieringa, 1936.

⁴ Barker, Ruben & Beck, 1940.

mechanism explains the fermentation of 1 mol. glucose to 2 mols. of acetic acid by *Cl. thermoaceticum*.¹ As Barker² suggested this fermentation is an oxido-reduction in which the CO₂ formed oxidises the intermediary products to acetic acid



This view was confirmed by the use of C¹⁴ added in the form of NaHC¹⁴O₃ to the culture medium;³ the C¹⁴ was found in both the methyl and carboxyl groups of the acetic acid and in the cells. Here the hexose is completely oxidised, CO₂ acting as the oxidising agent in a manner analogous to O₂ in aerobic oxidation. When pyruvic acid is the substrate the excess CO₂ accumulates²



Allied to the anaerobic decomposition of the purines is that of allantoin by *Str. allantoicus*.⁴ (Table 8.) This organism, besides fermenting a wide range of sugars, also brings about a rapid anaerobic decomposition of allantoin and appears to derive from this reaction the energy for growth as the other organic constituents of the medium (biotin and difco yeast extract) are present only in small amounts.

TABLE 8⁵
FERMENTATION OF ALLANTOIN

Products	mm/100 mm allantoin fermented
Ammonia . . .	226.0
Urea . . .	62.3
Oxamic acid . . .	44.8
CO ₂ . . .	168.0
Formic acid . . .	9.4
Acetic acid . . .	14.8
Glycollic acid . . .	13.8
Lactic acid . . .	1.5
Cell carbon . . .	13.1
Nitrogen recovery . . .	99.5%
Carbon recovery . . .	101.2%

The steps by which this fermentation occurs are not clear. Allantoin is probably first hydrolysed to urea and glyoxylic acid, the latter giving rise to oxalic acid (appearing as its amide, oxamic acid) by oxidation and glycollic acid by reduction.

¹ Fontaine *et al.*, 1941.

² Barker, 1944.

³ Barker & Kamen, 1945.

⁴ Barker, 1943 (2).

⁵ Barker, H. A., *J. Bact.*, **46**, 257, 1943.

CHAPTER III

POLYSACCHARIDES

POLYSACCHARIDES form the most important structural material of plants. Among bacteria they appear as capsules, as extracellular gums and, less often, as part of the internal cellular structure. In the form of capsules they are important to the cell as protection against invasive organisms. They may also function as reserve food material. To man they are of interest as conferring specificity on the bacterial antigens. They are evidently not essential to the life of the cell, as loss of ability to form capsules is a frequently occurring form of mutation and becomes apparent in the change of the smooth or mucoid colony to the rough or granular type.

Polysaccharides occur through the condensation of small molecules of the hexose type to form relatively insoluble hydrophilic gels composed of chains or networks of their constituent units. On hydrolysis the large molecules break down into their constituents; those most frequently found in bacterial polysaccharides are *d*-glucose, *d*-fructose, *d*-mannose, *d*-galactose, *d*-glucuronic acid, *d*-galactaronic acid, *d*-glucosamine and N-acetyl *d*-glucosamine.

Bacterial cellulose

Cellulose is comparatively rare among bacteria but was early recognised as a component of the acetobacter *A. xylinum* where it forms a slimy envelope.¹ More recently it has been isolated from this organism in a state of purity and shown both by chemical analysis and X-ray diffraction pattern to be identical with cotton cellulose;^{2, 3, 4, 5, 6} it has been found suitable for the production of osmometer membranes.⁷ The formation of cellulose depends on the presence of sugar or glycerol in the medium and is much increased by the addition of 0.5% ethanol, though it is not clear whether the latter acts by increasing growth or whether it has a specific action on cellulose production.⁸ Fructose is the preferred source of carbohydrate for cellulose production, though the

¹ Brown, 1886, 1887.

² Hibbert & Barsha, 1931.

³ Barsha & Hibbert, 1934.

⁷ Masson *et al.*, 1946.

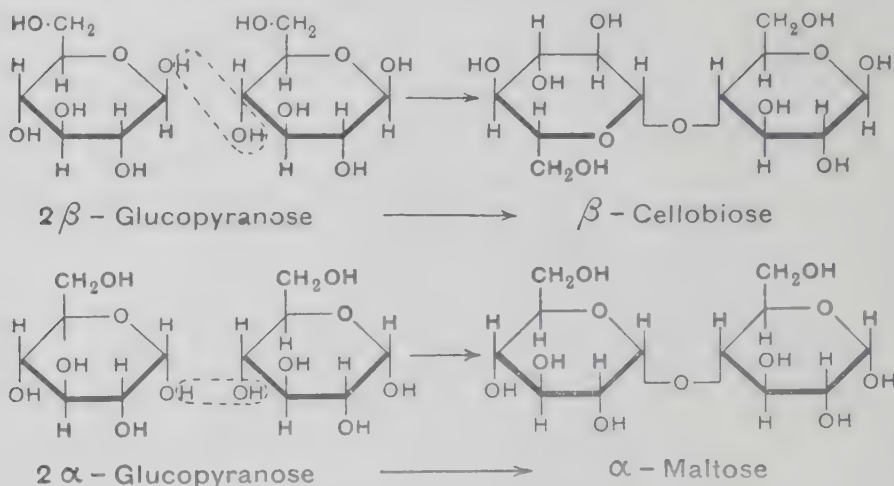
² Tarr & Hibbert, 1931.

⁴ Khouvine *et al.*, 1933.

⁶ Aschner & Hestrin, 1946.

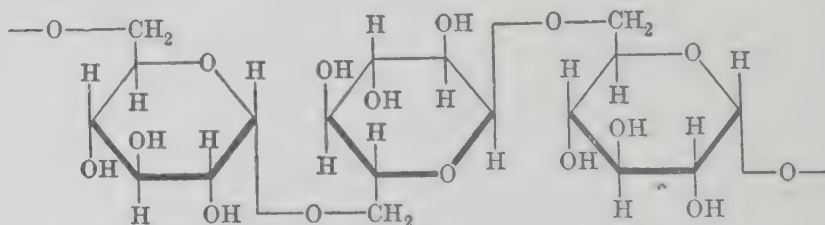
⁸ Tarr & Hibbert, 1931.

chemical nature of the cellulose is identical irrespective of the source of carbohydrate.¹



Dextrans and levans

Dextrans and levans are gums which on hydrolysis give glucose and fructose respectively. They were early noted by Pasteur, their production being termed by him "viscous fermentation." They occur as the result of infections in sugar factories, giving rise to waste and trouble in crystallisation. Both dextrans and



Dextran

levans are produced from sucrose but not from invert sugar or monosaccharides. Among the many organisms producing this type of polysaccharide are the following : dextrans by *Leuconostoc mesenteroides*,² *L. dextranicum*,³ *Betabacterium vermiforme* (Ward Meyer),⁴ *Phytophthora tumefaciens* A-6 (crown gall organism),⁵ *Beta-coccus arabinosaceus* Orla Jensen ;⁶ levans by *Bac. mesentericus*,⁷ *Bac. subtilis*,⁸ several soil *Actinomycetes* and unspecified organisms

¹ Barsha & Hibbert, 1934.

³ Peat *et al.*, 1939.

⁵ McIntire *et al.*, 1942.

⁷ Harrison *et al.*, 1930.

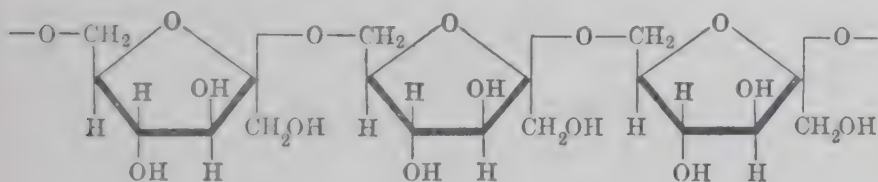
² Tarr & Hibbert, 1931.

⁴ Daker & Stacey, 1939.

⁶ Hassid & Barker, 1940.

⁸ Hibbert *et al.*, 1931.

from milk,¹ *B. polymyxa* (migula) and *Aer. levanicum*,² *Bac. mycoides*, *Ps. prunicola*, *Ps. mors prunorum*, *Ps. aptatum*,³ etc. The dextran formed by *L. dextranicum* and by *Betabacterium vermiforme* has been shown to consist of glucose units linked in the 1·6 position having a chain length of not more than 550 units.^{4, 5} The levan obtained by the action of *Bac. subtilis* and *Bac. mesentericus* was shown to be composed of units of 2 : 6 fructofuranose probably united through β -linkage.⁶ Levans based on the same unit are found in grasses; the molecular size of these is smaller than that of bacterial levans.⁷ The mode of production of levans and



Levan

dextrans has been recently found to be due to cell-free enzymes. In the case of three organisms the production of levan from sucrose has been shown to occur according to the equation^{8, 9}



In the case of *Bac. subtilis* the enzyme is adaptive and extra-cellular. When the organism is grown in a cellophane sack in peptone water with sucrose kept at a constant concentration of 10%, the levan formed within the sack reaches 12·5%. On agar the levan is found at a distance from the colonies, not as a capsule, and washed cells in sucrose synthesise levan actively. The enzyme could not be obtained from a Seitz filtrate, possibly owing to adsorption, but was recovered in a cell-free state from agar. In the case of the two other organisms studied, *Bac. polymyxa* and *Aer. levanicum*, the enzyme is constitutive and endocellular and the levan appears as a capsule.¹⁰ In the case of the latter organism the products of the reaction are levan, fructose and glucose; raffinose was broken down to levan, melibiose and fructose.¹¹

An analogous production of dextran was obtained by the action of a cell-free enzyme from *Leuconostoc mesenteroides* on sucrose:



In this case the intervention of a suggested phosphorylating phase was definitely disproved.¹² The production of the levan and

¹ Verbel, 1938.² Hestrin *et al.*, 1943.³ Cooper & Preston, 1935.⁴ Peat *et al.*, 1928.⁵ Baker & Stacey, 1939.⁶ Hibbert *et al.*, 1931.⁷ D. J. Bell, personal communication.⁸ Doudoroff, 1943.⁹ Hassid *et al.*, 1944.¹⁰ Hestrin *et al.*, 1943.¹¹ Hestrin & Alanen-Shapiro, 1944.¹² Hehre, 1943, 1946.

dextran has again been shown to be linked with the breakdown of sucrose and does not occur on mixtures of glucose and fructose.

Capsular polysaccharides

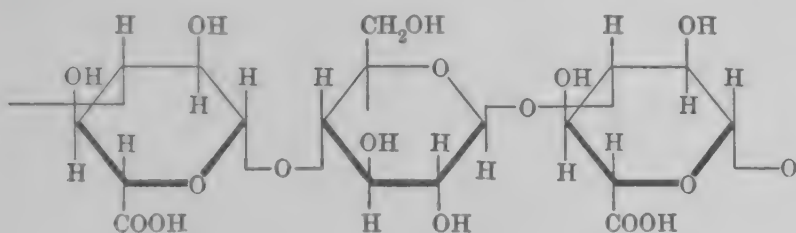
The majority of, but not all, bacterial capsules are composed of some form of polysaccharide. They are of great importance immunologically, conferring type specificity on closely related organisms such as the various types of pneumococcus. Though not themselves antigenic they are precipitated by serum at high dilution (e.g. $2 \cdot 10^3$) from an animal immunised with the homologous organisms, that is they act as haptens. Thus an animal immunised with pneumococcus type I produces a serum precipitating the polysaccharide of type I but not of types II or III. On the other hand, if the animal is immunised with the proteins of type I instead of with the intact cell a serum is produced which reacts with the proteins of the homologous organism but not with the intact cell nor with the free polysaccharide; moreover this serum is not specific, reacting equally well with proteins from other types of pneumococcus. Thus the proteins seem to be a necessary part of the antigen, whilst the polysaccharide confers the type specificity. Where the intact cells are used as antigen the serum reacts with free polysaccharide and is type specific; it does not, however, react with cell proteins.

The importance of bacterial polysaccharides in conferring specificity on bacterial antigens has greatly stimulated the study of their chemical composition. They are prepared from the culture medium by repeated precipitation with 50% ethanol, protein being eliminated at each step. Study of the thirty-two types of the pneumococcus shows that the structure of the capsular polysaccharide is probably the only difference between them. The polysaccharides have now all been obtained in a highly purified state.¹ Of these ten are N-free whilst five (I, IV, V, XII and XXV) have a significantly high content of nitrogen—about 5%. Acetyl groups are absent only in III and VIII. Nine types contain uronic acids and twenty contain amino-sugars. Types XXVIII and XXXII have a high phosphorus content—6.0 and 6.38% respectively—whilst eleven others have 3% and over.

The structure of the type III polysaccharide has been studied in detail. 300 l. of culture fluid gave 30–40 g. of a polysaccharide composed of 9.5% of glucose together with a disaccharide, 4- β -glucuronisido glucose or cellobiuronic acid. These units are linked

¹ Brown, 1939.

on position 3 of the glucuronic acid and position 4 of glucose, as shown.^{1, 2, 3, 4}



Glucuronic acid 1 : 4-glucose 1 : 3 glucuronic acid.

Polysaccharide of pneumococcus type III

The polysaccharides of *V. cholerae* and related vibrios found in water show some interesting relations. In type I, which includes most organisms from cholera cases, the polysaccharide was hydrolysed, giving galactose and an aldobionic acid consisting of galactose and glucuronic acid. In type II, which includes a few vibrios from cholera cases and also all the non-pathogenic water vibrios tried, the polysaccharide gave arabinose and an aldobionic acid consisting of galactose and glucuronic acid. Type III was obtained as follows: a dissociant from a cholera vibrio of type I, known as Rangoon smooth, gave rise to a rough colony consisting of non-agglutinating organisms the polysaccharide of which was identical with that of the parent but only one-fifth in amount; from this strain a second variant with a different colony formation was isolated called Rangoon rough II; this had the same amount of polysaccharide as the original parent but of a different composition, giving only glucose on hydrolysis. The same polysaccharide appeared also in a vibrio from a cholera case (Basrah II).⁵ Subsequent work has shown that all three polysaccharides exist in the cell in an acetylated form and contain nitrogen;⁶ the acetyl group is split off by the use of alkali in the extraction as in the case of pneumococcus I.

Data on the chemical structure of bacterial polysaccharides are accumulating and it is impossible to give a comprehensive account of the subject here; the reader is referred to the reviews which appear frequently.⁷

Enzymic decomposition of polysaccharides

Starch, cellulose, glycogen, chitin, animal and plant polysaccharides, as well as those of microbial origin, are all broken down

¹ Heidelberg & Goebel, 1926, 1927.

² Heidelberg & Kendall, 1932.

³ Hotchkiss & Goebel, 1937.

⁴ Reeves & Goebel, 1940.

⁵ Linton & Miles, 1934.

⁶ Heidelberg, Kendall & Scherp, 1936.

⁷ Evans & Hibbert, 1946.

by some organism or another, usually by adaptive exocellular enzymes. The method of isolating organisms with the necessary enzymes for hydrolysing any given polysaccharide has been formulated by the classical work of Dubos. It rests on the fact that all naturally occurring organic compounds left in the soil ultimately disappear, though at very different rates. This is due to enzymes of microbial origin. It is, however, obvious that in the case of rarer compounds such as a type-specific polysaccharide, the power to bring about its decomposition is not likely to have much survival value for the organism possessing it. In order to find such organisms amongst the mixed population of the soil it is therefore necessary to adjust conditions favouring their multiplication. This is done by inoculating a medium in which the pure compound in question forms the sole source of carbon with soils from selected localities. In time a culture is obtained in which an organism with the required enzyme predominates; this is subsequently isolated by the usual methods. Such enzymes are generally adaptive and filterable.

The breakdown of starch

Starch and glycogen are polysaccharides consisting of chains of α -glucopyranose units united in the 1:4 positions. Starch is hydrolysed by amylases to smaller units and ultimately to the disaccharide maltose, which is further hydrolysed to glucose by maltase. Several amylases of plant origin exist which break down starch to shorter chains—dextrins—which differ in their reaction with iodine to give coloured products, and in their reducing properties.¹ Thus barley contains a β -amylase which decomposes starch to maltose together with a residue of about twelve units giving a blue colour with iodine, and an α -amylase which gives dextrins with little or no reducing power forming no coloured compounds with iodine. Probably the amylases of bacteria belong to these as well as to other types.

The breakdown of starch by *Cl. acetobutylicum* has been shown to be due to two enzymes, an amylase and a maltase. Both enzymes are obtained in the culture filtrate when the organism is grown on maize meal. When grown on yeast autolysate with maltose, maltase only is formed; neither enzyme is produced when sucrose or glucose replaces maltose. The amylase was concentrated by evaporation, dialysis, precipitation with $(\text{NH}_4)_2\text{SO}_4$, adsorption on to starch and elution with phosphate buffer at pH 5.8. This procedure removed 95% of the maltase. The enzyme thus purified converts starch to maltose together with some reducing sub-

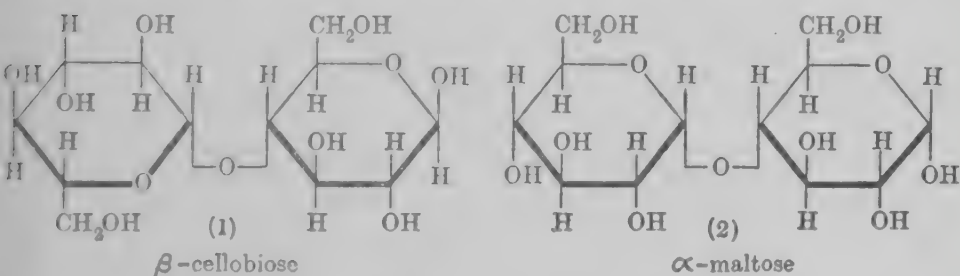
¹ Hanes, 1937.

stances, probably dextrans, not completely eliminated ; the maltase converts maltose quantitatively to glucose.¹

A special type of crystalline dextrin obtained by the action of *Bac. macerans* on starch was first described by Schardinger.² The dextrans thus obtained were of two types with differing crystalline form and solubilities. They gave crystalline compounds with iodine. The amylase concerned was subsequently obtained cell-free and purified by precipitation with acetone ;³ the dextrans are non-reducing.

The breakdown of cellulose

Probably owing to the fact that its unbridged molecular chains are almost entirely free from hydroxyl groups as such, cellulose is more resistant to hydrolytic attack, whether enzymic or chemical, than is starch. Like the latter it is hydrolysed first to a disaccharide—cellobiose—and finally to glucose. The configuration of cellobiose and maltose respectively is shown in 1 and 2 below.



Cellulose consists of long chains of cellobiose units, the number which is uncertain.

It is decomposed enzymatically by two enzymes, cellulase, which breaks it down to cellobiose, and cellobiase, which attacks cellobiose with production of glucose.

The separate action of cellulase and cellobiase was first shown by Pringsheim in the bacterial decomposition of filter paper. Cellobiose was obtained by stopping the fermentation when glucose first appeared and was identified by the ozazone. Glucose was obtained by the addition of calcium nitrate after the fermentation had well started ; this arrested the further decomposition of the glucose whilst allowing its formation from cellobiose to proceed.⁴ The same effect can be brought about by toluene.⁵ As Pringsheim was probably working with a mixed culture it is possible that cellulase and cellobiase were in different organisms. This doubt does

¹ Hockenhull & Herbert, 1945.

² Tilden & Hudson, 1939.

³ Woodman & Stewart, 1928.

⁴ Schardinger, 1909.

⁵ Pringsheim, 1912.

not, however, arise in the work of Simola¹ who, using a pure culture of an aerobe, *Cellulobacillus myxogenes*, showed the formation of cellobiose and glucose. He grew the culture on a medium containing nutrient salts with Liebig's meat extract and peptone, chalk and filter paper (Medium I). After 8 days' growth at 37° the paper and adhering organisms from 6 l. of medium were centrifuged and washed and resuspended in 500 ml. of tap water and phosphate buffer pH 6.0, with 10 ml. of toluene. After 8 days' further incubation the fluid was separated from the residue and evaporated at 40°. The residue, which was highly reducing, was extracted with alcohol. Glucose, amounting to 102 mg., was identified in the alcoholic extract. Cellobiose amounting to 346 mg. was found in the residue and identified by the specific rotation ($[\alpha]_D^{20} = 35.0^\circ$; cellobiose = 34.6) and by the ozazone. It was not fermented by yeast but was hydrolysed by emulsin; its identity with cellobiose was fully established.

Organisms containing cellulase are very widespread in the soil and in the gut of herbivores and in all places where decaying plant material is found. They are isolated on plates containing finely divided cellulose as sole source of carbon, or in appropriate liquid media.

The separation of a cell-free cellulase is not as easy as in the case of the amylases. The cellulase organisms usually adhere closely to the insoluble cellulose fibres, which gradually disintegrate. Simola, however, demonstrated the presence of cellulase in the culture fluid of the aerobe *Cellulobacillus myxogenes* in the following way. In each of three flasks were placed 50 ml. water, 90 g. filter paper impregnated with bacteria and 20 ml. toluol. The flasks were incubated for 2, 3 and 5 days respectively and the bacteria, etc., then removed by filtration. The fluid was then mixed with finely divided cellulose, phosphate buffer at pH 6.0 and toluol. Reducing substances, absent at the beginning, appeared in 3 and 5 days. It is uncertain whether the cellulase is in the form of a filterable enzyme or produced by autolysis; in any case it is strongly adsorbed to the cellulose. A cell-free cellobiase was demonstrated by the same method; this enzyme has also been found in malt extract.^{2, 3}

Cytophaga I attacks cellulose aerobically forming a mucilage containing xylose and CO₂.⁴ The report that it is unable to use glucose is incorrect.⁵

¹ Simola, 1931.

² Ibid.

³ Pringsheim & Leibowitz, 1923.

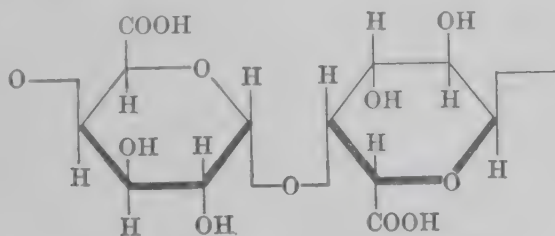
⁴ Walker & Warren, 1938.

⁵ Stanier, 1942.

Amongst the anaerobes cellulose breakdown is associated with different types of fermentation. *Cl. cellulosolvens* from horse faeces attacks, besides cellulose, only dextrin, arabinose and xylose, the products being CO_2 , H_2 and organic acids. Glucose is not attacked.¹ *B. cellulosa dissolvens*, isolated from the human gut, gives a butyric fermentation;² an organism from cattle rumen gives a mixed fermentation of the *coli* type;³ an anaerobic *actinomyces* from termite gut and from the rumen gives a propionic fermentation.⁴ Cellobiose can be demonstrated where fermentation is inhibited but glucose is never shown.

*Pectin and other plant polysaccharides*⁵

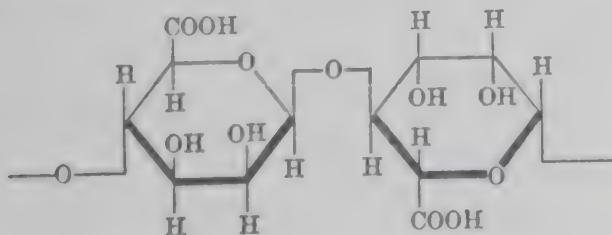
Pectic substances form a high proportion of the interlamellar material of plants. They consist of arabans, galactans and pectic acid. The last-mentioned has been shown to be composed of β -galacturonic acid residues united through the 1:4 positions. The proportions in which these three polysaccharides occur vary in different plant pectins.



Pectic acid. β -1:4-galacturonic acid

Pectins proper are methyl esters of pectic acid; they are readily broken down by bacterial action. Industrially this process is used in freeing the cellulose fibres in the retting of flax; it also occurs in the rotting of fruit and vegetables and assists the invasive action of plant pathogens.

The polysaccharide corresponding to pectic acid in seaweeds is alginic acid composed of mannuronic acid linked in the 1:4 positions.



Alginic acid. β -1:4-mannuronic acid

¹ Condon & Penger, 1931.

² Khourine, 1923.

³ Hungate, 1944.

⁴ Ibid., 1946.

⁵ Hirst, 1942.

Alginic acid is readily attacked by marine and soil bacteria. A cell-free alginase has been described; this is stated to break down alginic acid to smaller units, but not to free mannuronic acid.¹

Breakdown of hyaluronic acid

This important mucopolysaccharide is a constituent of skin, aqueous humour, synovial fluid, umbilical cord and Wharton's jelly. It is composed of equal parts of glucuronic acid and N-acetylglucosamine. It is hydrolysed by hyaluronidase (the "spreading factor") present in the filtrates of a number of pathogenic organisms, e.g. *Cl. welchii*, *Cl. septicum*, *Str. pyogenes* and *Staph. aureus*. The enzyme has been partially purified and shown to act specifically on hyaluronic acid. Its first action is to lower the viscosity of hyaluronic acid, after which glucuronic acid, glucosamine and subsequently acetic acid and ammonia are liberated.

In vivo hyaluronidase increases the invasiveness of pathogenic organisms, particularly those of the gas gangrene group, by decomposing the cementing substance occupying the interstitial spaces of the skin.^{2, 3, 4}

Enzymic decomposition of specific polysaccharides

Enzymes hydrolysing the specific polysaccharides have been found to occur in quite unrelated organisms. Thus Avery and Dubos⁵ isolated a soil organism from bog peat by repeated reinoculations into media in which the polysaccharide of pneumococcus III formed the sole source of carbon (see also Chapter XI, p. 295). This organism, when grown in the presence of the polysaccharide or of the aldobionic acid derived from it, excretes an enzyme which hydrolyses the polysaccharide in question, but is inactive on related substances. The enzyme preparation obtained by filtration decomposes the polysaccharide, not only when purified, but also in the capsular condition with the intact cell both in broth culture and in mice; thus when injected into mice infected with type III it affords protection.⁶

Organisms isolated from soil by a similar method have been found to decompose the specific polysaccharides of types I,⁷ II⁸ and III.⁹ The organism attacking the polysaccharide of type VIII is without action on that of type III in spite of the chemical similarity of these substances.

Change from one type to another

It was first shown by Griffith¹⁰ that if an R (unencapsulated)

¹ Waksman *et al.*, 1934. ² McLean, 1941. ³ Meyer & Palmer, 1936.

⁴ East *et al.*, 1941.

⁵ Avery & Dubos, 1931.

⁶ *Ibid.*

⁷ Sickles & Shaw, 1934.

⁸ *Ibid.*, 1933.

⁹ *Ibid.*, 1935.

¹⁰ Griffith, 1928.

strain of type II pneumococcus was inoculated subcutaneously into a mouse along with a large dose of an S (encapsulated) form of type I, previously heat-killed, the organism recovered was encapsulated type I. This work was extended by Dawson and Sia,¹ who dispensed with the mouse and produced the change *in vitro*. Alloway² further simplified the system by using as his transforming agent heated filtered extracts of cells or extracts of cells disrupted with 10% desoxycholate. The final and highly significant simplification is contained in the work of Avery, McLeod and McCarty.³ These workers used again the R unencapsulated strain of type II which by the methods described had already been transformed into types III, VI and XIV. This organism was grown on charcoal-treated nutrient broth with serum heated to 60-65°. The transforming agent was prepared from type III. The cells from 50-75 l. of heart broth were removed and disrupted; the proteins and polysaccharides were removed and the residue purified by repeated alcohol precipitation. The final product was a polymer of desoxyribose nucleic acid. The elementary analysis was correct; its activity was destroyed by the depolymerase specific for that substance. It was homogeneous in the ultracentrifuge, and in the Tiselius apparatus only one substance of high electrophoretic mobility was found. It was active in a concentration of 1.6×10^7 . The significance of this highly important observation will be discussed under variation.

¹ Dawson & Sia, 1931.

² Alloway, 1932, 1933.

³ Avery *et al.*, 1945.

CHAPTER IV

FERMENTATION

FERMENTATION is the anaerobic decomposition of carbohydrate and related compounds to products which cannot be further decomposed by the enzyme system or cell except by the intervention of molecular O_2 . These fermentation products differ in different organisms, being governed in the main by the battery of enzymes at the disposal of the cell and to a smaller extent by conditions. The biological importance of fermentation consists in the provision of a source of energy for the anabolic processes of the growing cell and it is noteworthy that fermentations which differ widely in their end products approximate closely in the amount of energy made available.

The breakdown of carbohydrate does not occur in one step or two but consists of a series of well-defined reactions each catalysed by its own enzyme. These reactions are of two kinds, oxidoreductions and phosphate transference, the former providing the energy and the latter the mechanism of its transfer.

The two commonest forms of fermentation are those first studied by Pasteur, viz. lactic and alcoholic. The former is found in most groups of living organisms; the latter is characteristic of yeasts, but is found also in plant tissues and occasionally in moulds and bacteria. These two fermentations proceed along the same path to the penultimate stage where their divergence is due to the presence or absence of a special enzyme, carboxylase, characteristic of yeast cells.

The intermediate steps of fermentation have been studied most intensively in yeast (alcoholic) and muscle (lactic) fermentations. In order to follow the more recent work on bacterial fermentations it is desirable to get a clear view of the present knowledge on the fermentation by yeast. It is not proposed to treat this historically, as would be demanded if this book were on yeast metabolism, but instead to give a summary of the present position and thence to pass to a more detailed study of bacterial fermentations. The scheme detailed below is due mainly to the work of the Embden, Meyerhof and Parnas schools, later reinforced by the work of the Cori, and will be referred to as the E.M.P. scheme.

The oxidoreduction reactions are catalysed each by a separate enzyme. These enzymes have now all been prepared from yeast

and all act in conjunction with coenzyme I (see p. 18); some also require a metal, Mg or Mn or another heavy metal. The phosphorylations and their reverse processes are brought about by another group of enzymes transferring phosphate groups instead of hydrogen. Just as in the case of hydrogen, phosphate transfer requires an enzyme and a carrier to act as a phosphate acceptor or donator. The latter part is played by adenosine, which can carry one, two or three phosphate groups in the three phosphorylated forms, adenosine monophosphate (adenylic acid), adenosine diphosphate (A.D.P.) and adenosine triphosphate (A.T.P.) (see Fig. 1). These esterifications are all endothermic, adenosine

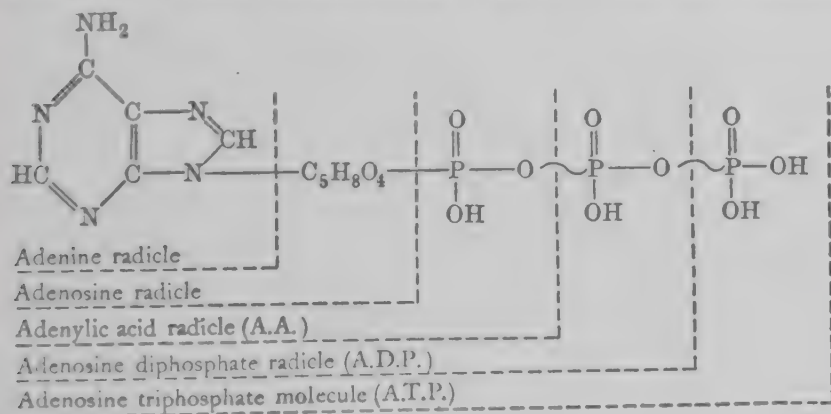


FIG. 1

→ A.A. requiring approximately 3000 cals., A.A. to A.D.P. 9000-11,000 cals. and A.D.P. → A.T.P. 9000-11,000.¹ The fact that in the two latter phosphorylations a large amount of energy is absorbed is denoted by the use of the symbol ~ in the O ~ P linkage.

The complete series of reactions are schematically represented in Fig. 2; the arabic numerals on the left of or above the arrows refer to the enzymes and the letters on the right of or below to the coenzymes.

Sugar must be phosphorylated preliminary to fermentation. This may occur in two ways: (1) the starch, glycogen or other form of polysaccharide may undergo phosphorolytic breakdown (comparable to hydrolytic breakdown) by which one molecule of 1-phosphoglucose (Cori ester) is split off by the enzyme phosphorylase (1), inorganic phosphate being the phosphorylating agent;² or (2) glucose may be phosphorylated direct to 6-phosphoglucose by the enzyme hexokinase (16), A.T.P. being the phosphorylating agent. In the former case 1-phosphoglucose undergoes an enzy-

¹ Lipmann, 1941.

² Cori & Cori, 1936.

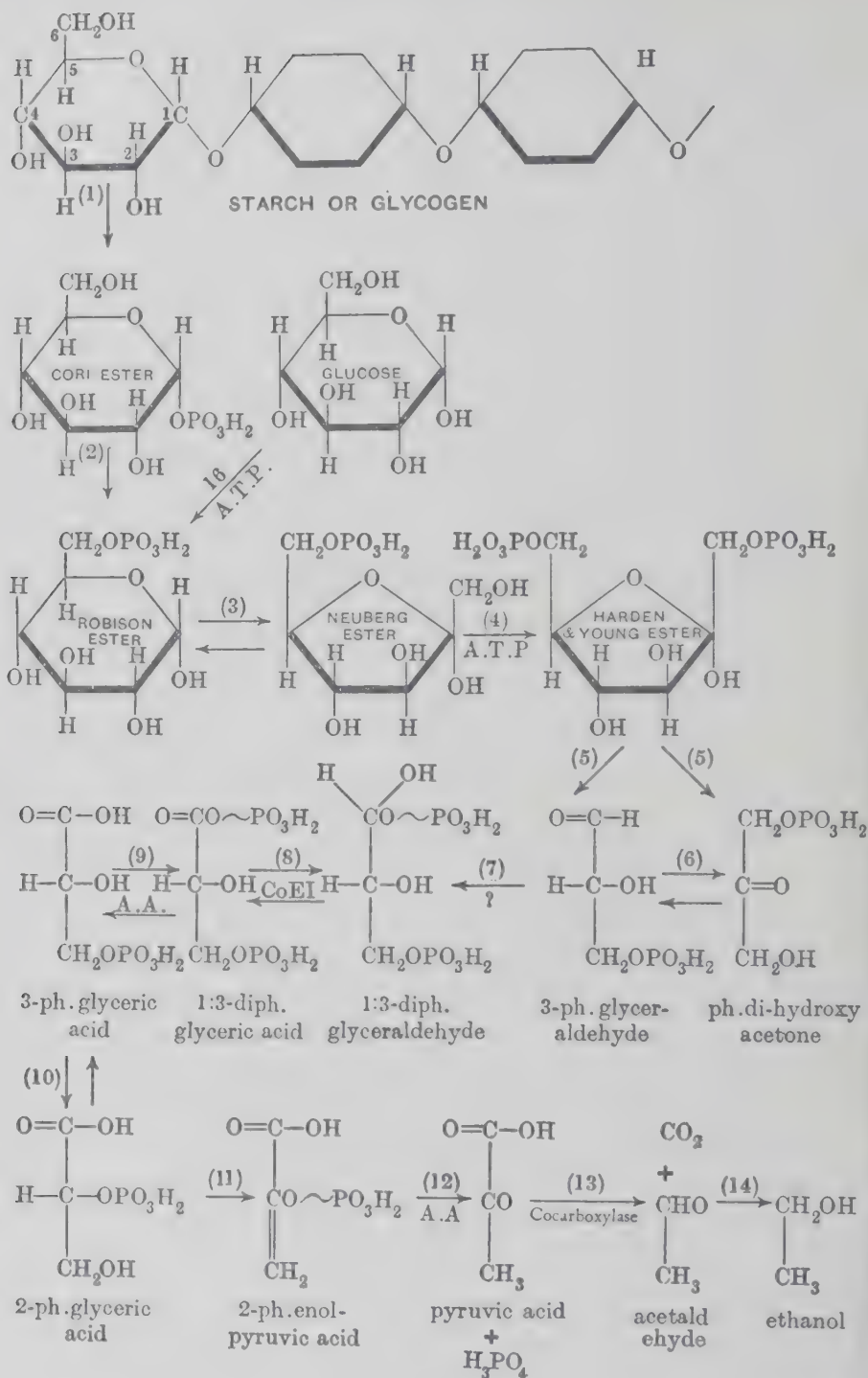


FIG. 2

mic shift of the phosphate from position 1 to position 6, 6-phosphoglucose (Robison ester) being formed as in the direct phosphorylation of glucose. This enzyme—phosphoglucumutase (2)—requires Mg or Mn for its activity;¹ actually an equilibrium is set up between the Cori and Robison esters, about 5% of the former and 95% of the latter being present. The Robison ester then undergoes another internal shift—enzyme phosphohexose isomerase (3)—to phosphofructofuranose (fructose monophosphate, Neuberg ester).² This in turn is phosphorylated by an enzyme, phosphohexokinase (4),³ acting in conjunction with A.T.P. to give 1:6-diphosphofructofuranose (the hexosediphosphate of Harden and Young).

The hexose transformations now come to an end. The next change is a split into two 3-carbon phosphorylated compounds, 3-phosphoglyceraldehyde and phosphodihydroxyacetone, the enzyme catalysing this change being known as zymohexase or aldolase (5);^{4, 5} The enzyme preparation was made from muscle⁶ and also from yeast,⁷ the latter being activated by Zn, Co, Cu and Fe. A second enzyme, phosphotriose isomerase (6), shifts the equilibrium proportion of these two substances in favour of phosphodihydroxyacetone.⁸ The 3-phosphoglyceraldehyde is, however, the compound on the main path of the glycolysis and is again phosphorylated, producing 1:3-diphosphoglyceraldehyde, the phosphorylating agent being inorganic phosphate and the enzyme unknown (this reaction may possibly be non-enzymic).

The next reaction is an oxidoreduction by the enzyme triose-phosphate dehydrogenase (8) involving coenzyme I,⁹ resulting in the formation of 1:3-diphosphoglycerate + reduced coenzyme I. In the presence of A.A. or A.D.P. the labile phosphate is transferred, forming A.T.P. and 3-phosphoglyceric acid.

The reduced coenzyme I couples this oxidation with the reduction of acetaldehyde to ethyl alcohol (14) and the A.T.P. formed is available for the phosphorylation reactions catalysed by enzymes (4) and (16).¹⁰ The next reaction involves an internal shift of the phosphate group, by triosemutase—or phosphoglyceromutase (10)—3-phosphoglycerate → 2-phosphoglycerate. This enzyme depends on the presence of heavy metals.

2-Phosphoglycerate is converted into phosphoenolpyruvate by enolase¹¹ (11) and (12), activated by Mg and inhibited by fluoride. This is again a double reaction in which phosphoenolpyruvic undergoes an enol-keto transformation and a dephosphorylation

¹ Cori *et al.*, 1938.

² Lohmann, 1933.

³ Ostern *et al.*, 1936.

⁴ Warburg & Christian, 1942.

⁵ Meyerhof & Lohmann, 1934.

⁶ Herbert *et al.*, 1940.

⁷ Warburg & Christian, 1942.

⁸ Meyerhof & Lohmann, 1934.

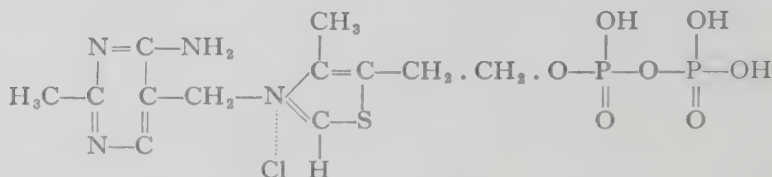
⁹ Warburg & Christian, 1939 (1), (2).

¹⁰ Needham & Palla, 1937.

¹¹ Meyerhof & Lohmann, 1934.

in the presence of a phosphate acceptor, the energy liberated in the former reaction being used for the esterification of adenosine to A.A. or of A.A. to A.D.P. or of A.D.P. to A.T.P. K, Mn (or Mg) are also necessary for the phosphate transfer.¹

Pyruvic acid is then decarboxylated (13) to $\text{CO}_2 + \text{CH}_3 \cdot \text{CHO}$ by the enzyme carboxylase (especially characteristic of yeast) acting in conjunction with cocarboxylase; the $\text{CH}_3 \cdot \text{CHO}$ is reduced to ethanol by the reduced coenzyme I formed in reaction 8.



Cocarboxylase, the diphosphate of aneurin (vitamin B₁)

The energy shifts in the series of enzyme changes must now be considered; these will be dealt with very briefly and the reader is referred to the classical paper by Lipmann² for a full treatment of the subject.

The esterification of inorganic phosphate with an alcoholic hydroxyl has been computed to require about 3000 calories. This type of phosphate linkage is known as "energy poor" and is written in the usual way. The following esters are of this type: the phosphates of hexose, glycerol, acetaldehyde, dihydroxyacetone and adenylic acid. Other types of phosphate involving P-OP as in A.D.P. and A.T.P., carboxylphosphate as in 1:3-diphosphoglyceric acid, enolphosphate as in phosphoenolpyruvate, are known as "energy rich" linkages (written $\text{O} \sim \text{P}$) and have been computed to require 9000–11,000 cal. for their formation. Hence formation of an energy-rich linkage of these types can occur only if linked to a highly exergonic reaction. Thus the change from 3-phosphoglyceraldehyde to 1:3-diphosphoglyceric acid (via 1:3-diphosphoglyceraldehyde) occurs only when accompanied by an oxidoreduction of phosphoglyceraldehyde to phosphoglyceric acid balanced by a reduction of acetaldehyde to alcohol. The energy liberated in oxidoreduction can in this way be stored in the energy-rich $\sim \text{P}$ linkage.

Our knowledge of the metabolic uses of the energy stored in the $\text{P} \sim$ bond is at present fragmentary. Muscular contraction has been shown to be associated with the change $\text{A.T.P.} \rightarrow \text{A.D.P.}$, the enzyme for which is identical or closely associated with the structural protein of muscle^{3, 4} itself. Phosphate bond energy is used for the synthesis of starch and glycogen, since the phosphorylation

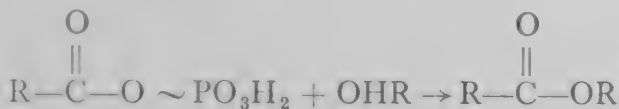
¹ Boyer *et al.*, 1943.

³ Ljubimova & Engelhard, 1941.

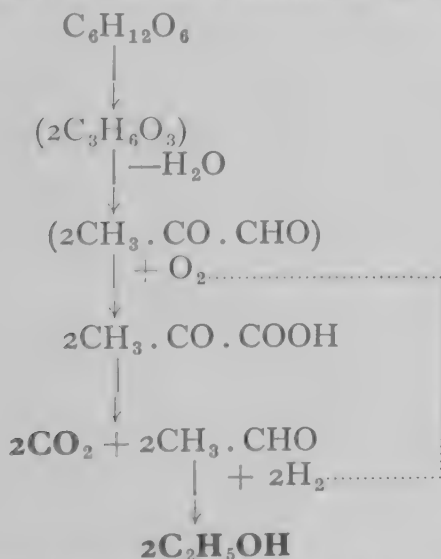
² Lipmann, 1941.

⁴ Needham *et al.*, 1941.

of glucose by A.T.P. and hexokinase is a necessary preliminary to its conversion to polysaccharide by phosphorylase. It is probable also that the synthesis of fats occurs through the formation of acyl bonds by the interaction of A.T.P. with —COOH to form acyl linkages which readily esterify.¹



Before leaving the subject of yeast fermentation it is necessary to refer to an earlier scheme put forward by Neuberg which, prior to the discovery of the phosphorylation cycle, dominated fermentation chemistry for more than a decade, though now mainly of historic interest. This scheme was developed by observations made on intact yeast rather than yeast juice and is an attempt to explain the process by a series of hypothetical intermediates. According to this view the Gay-Lussac equation $\text{C}_6\text{H}_{12}\text{O}_6 = 2\text{C}_2\text{H}_5\text{OH} + 2\text{CO}_2$ is arrived at according to Scheme I.

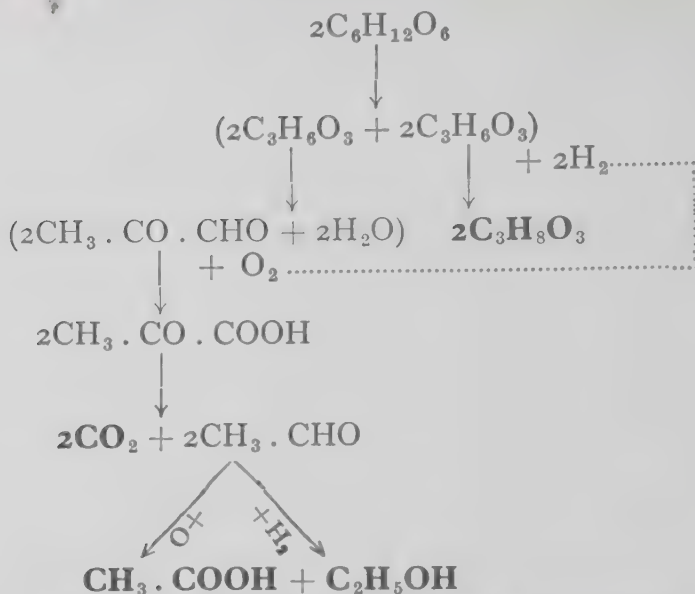


Scheme I (Neuberg's "first form" of fermentation)

If the reaction of the medium is alkaline, the course of the fermentation is modified; a second hydrogen acceptor then becomes active and competes with acetaldehyde for the hydrogen available when the methyl glyoxal becomes oxidised; this second hydrogen acceptor was not identified, but was regarded as an unstable compound of the formula $\text{C}_3\text{H}_6\text{O}_3$ (Scheme II) since its reduction product appeared as glycerol. The acetaldehyde, therefore, instead

¹ Lipmann, 1941.

FERMENTATION



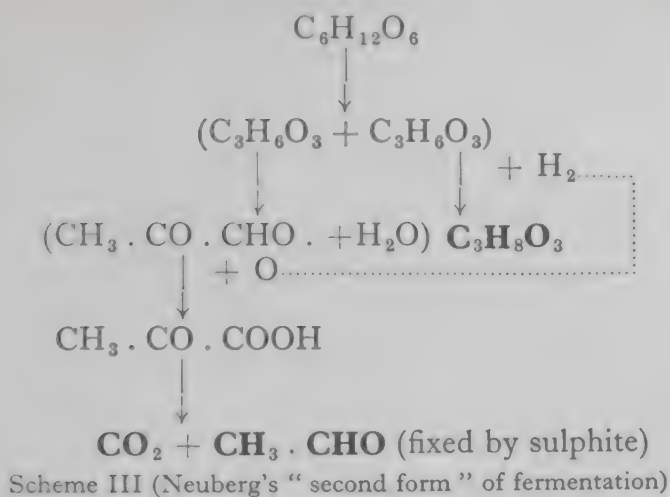
Scheme II (Neuberg's "third form" of fermentation)

of becoming wholly reduced as in Scheme I, was believed to undergo a Cannizzaro reaction in which half the molecules were oxidised to acetic acid and half were reduced to ethyl alcohol. Such a fermentation is represented in Scheme II (Neuberg's "third or dismutation form" of fermentation).¹

Finally Neuberg was able to prove the occurrence of acetaldehyde in the course of the fermentation by the addition of sulphite to the medium. In these circumstances the sulphite present combines with part of the acetaldehyde before this has been oxidised or reduced; at the end of the fermentation the acetaldehyde so fixed can be recovered, identified and estimated. With the removal of the acetaldehyde it is clear that a hydrogen acceptor disappears from the system as it is given in Scheme I; hence, as in Scheme II, this is replaced by the substance $\text{C}_3\text{H}_6\text{O}_3$, which by reduction passes to glycerol. Thus for every molecule of acetaldehyde fixed by the sulphite a corresponding molecule of glycerol was to be expected. This was realised; throughout the fermentation glycerol and acetaldehyde appear in equimolecular proportions.² Such a fermentation is depicted in Scheme III (Neuberg's "second or fixation form" of fermentation):

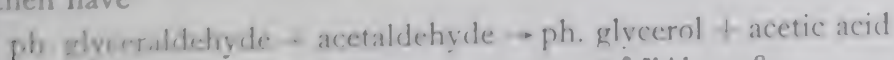
It must be pointed out, however, that none of the three schemes was ever realised in its entirety. Scheme I approaches fulfilment most nearly, but even here glycerol and acetic acid always occur to a small extent. In the fermentation carried out in the presence of

¹ Neuberg & Hirsch, 1919 (1).² Ibid. (2).



sulphite a proportion only conforms to Scheme III, the actual process being a mixture of those depicted in Schemes III and I. Scheme I rested first on the fact that pyruvic acid can be shown to be an intermediate product in yeast fermentation, by the use of dried yeast on magnesium hexose diphosphate,¹ and that pyruvic acid is fermentable at a rate equal to that of glucose. Secondly, that a $\text{C}_3\text{H}_6\text{O}_3$ compound (methyl glyoxal) can be isolated in special circumstances, viz. fermentation of magnesium hexosediphosphate by extract of dried bottom yeast with toluene water, this being known as Neuberg's fourth form of fermentation.² Neuberg regarded this methyl glyoxal as an intermediate product of fermentation accumulating as a result of the special conditions used.

The fact that methyl glyoxal is not fermentable by yeast was a serious objection to this scheme but was overlooked by regarding methyl glyoxal as an inactive stabilisation product of a more active substance (a subterfuge frequently resorted to when facts fail to fit biochemical theories). The accumulation of acetaldehyde in the presence of sulphite is equally explicable on the E.M.P. scheme and the simultaneous accumulation of glycerol would fit in with the removal of $\text{CH}_3 \cdot \text{CHO}$ as a hydrogen acceptor and the alternative reduction of triosephosphate to glycerol phosphate, followed by the hydrolysis of the latter. The production of equimolecular amounts of glycerol and acetic acid in the alkaline medium (Neuberg's third form of fermentation) might be explained on the change in pH affecting the course of the oxidoreduction; instead of ph. glyceraldehyde + acetaldehyde \rightarrow ph. glyceric acid + ethanol, which occurs at neutral and acid reactions, we should then have



¹ Neuberg & Kobel, 1929.

² Ibid., 1928.

the parts of oxidant and reductant being reversed ; this would be followed by the hydrolysis of phosphoglycerol to phosphate and glycerol.

The methylglyoxal of Neuberg's " fourth form of fermentation " is not necessarily a true intermediate since it has been found to occur by the acid hydrolysis of triosephosphate.¹ In any case the occurrence or non-occurrence of methylglyoxal in fermentation raises the interesting problem of glyoxalase. This enzyme catalyses the change



glutathione being the coenzyme. This enzyme is one of the most widely distributed known,² yet its substrate is never met with except in the artificial conditions mentioned above ; until these facts are reconciled we cannot be certain that lactic acid arises invariably by the reduction of pyruvic.

Fermentation by the Enterobacteriaceæ

The two tribes mainly studied have been the *Escherichææ* and the *Salmonelleæ*, organisms previously included in the *coli-typhosus* group.

The products in these fermentations are so numerous that no single one predominates sufficiently to characterise the fermentation. Harden³ made the first quantitative estimation of the products of a *coli* fermentation and attempted to construct a picture of the breakdown process. He found the following products : lactic, acetic, formic and succinic acids, alcohol, CO_2 and H_2 , and fairly successfully accounted for the sugar fermented in the products of fermentation.

Fructose, galactose and arabinose gave the same products in approximately the same proportion as glucose ; the corresponding alcohol, mannitol, gave more ethyl alcohol and less acetic acid and lactic acids.

The appearance of ethyl alcohol and acetic acid in approximately equimolecular proportions led Harden to postulate their origin from a common precursor. Later Neuberg⁴ showed the occurrence of acetaldehyde by sulphite fixation and deduced that this was an intermediate. Kay, following up the work of Harden, showed that reduction and oxidation products of the hexoses are fermented along the same lines, the former giving rise to more reduction and the latter to more oxidation products.⁵

Valuable quantitative studies of this and other fermentations have been made by the Delft school.⁶ These have been inter-

¹ Meyerhof & Lohmann, 1934.

² Hopkins & Morgan, 1945.

³ Harden, 1901.

⁴ Neuberg & Nord, 1919 (1).

⁵ Kay, 1926.

⁶ Scheffer, 1928.

interpreted along the lines of the Neuberg scheme then currently accepted; modern work renders this interpretation unlikely but the work provides the most complete and reliable quantitative data on fermentations in growing cultures.

TABLE 1

PRODUCTS OF THE FERMENTATION OF VARIOUS COMPOUNDS BY *B. coli*
(AS PERCENTAGE OF COMPOUND FERMENTED)

	Mannitol (Grey)	Glucose	Gluconic acid	Glycuronic acid	Saccharic acid
Lactic acid .	24.6	44.6	35.1	17.5	5.8
Succinic acid .	6.9	5.0	9.6	14.6	15.5
Acetic acid .	7.0	16.8	23.4	48.2	49.1
Formic acid .	7.4	0.8	0.5	0.6	2.5
Carbon dioxide	27.3	12.4	13.8	5.0	22.0
Ethyl alcohol .	27.0	16.1	10.8	2.6	1.0
Total .	100.2	95.1	93.2	88.5	95.9

In addition to the products already mentioned it was early noticed¹ that certain members of the group produced acetylmethylcarbinol (acetoin) and its reduction product 2 : 3-butylene glycol $\text{CH}_2 \cdot \text{CO} \cdot \text{CHOH} \cdot \text{CH}_3$ and $\text{CH}_3 \cdot \text{CHOH} \cdot \text{CHOH} \cdot \text{CH}_3$). Acetoin was first attributed to the condensation of 2 mols. of $\text{C}_2\text{H}_5 \cdot \text{CHO}$, as it was found that the addition of this substance to bacterial² and yeast³ fermentations increased acetoin production. Early also was the discovery that the well-known reaction of Voges and Proskauer⁴ was due to the oxidation of acetoin and 2 : 3-butylene glycol to diacetyl $\text{CH}_3 \cdot \text{CO} \cdot \text{CO} \cdot \text{CH}_3$ and the condensation of the latter with guanine derivatives.⁵

Application of the E.M.P. scheme to coli fermentation

Advance in knowledge of bacterial fermentation lagged behind that of yeast owing to the difficulty of making cell extracts comparable to yeast and Lebedev juice. This difficulty has now been overcome (see p. 16) and the intermediate reactions in this group of fermentations are now fairly clear.

As in other fermentations the intermediate steps have been elucidated by the use of cell preparations (acetone powders, toluene-treated cells, etc.), cell extracts and the use of specific inhibitors. Tikka⁶ first showed that washed suspensions of *coli* fermented glucose and hexosediphosphate to the same end products and that both fermentations are influenced in the same way by changes in pH, more acetic and less lactic acid being produced

¹ Harden & Wolpole, 1906.

² Neuberg & Hirsch, 1921.

³ Harden & Norris, 1912.

⁴ Harden & Norris, 1912.

⁵ Voges & Proskauer, 1898.

⁶ Tikka, 1935.

at pH 7.3-7.6 than at 6.02-6.25. In the absence of inhibitors phosphoglycerol gave ethanol (78% yield) and phosphoglyceric acid gave acetic acid; formic acid was assumed (but not proved) to be the other product in each case. Phosphoglyceric acid in presence of toluene gave pyruvic, which in the absence of the inhibitor gave acetic, formic and lactic acids. Using acetone preparations of *coli* hexose was fermented mainly to pyruvic acid, toluene and acetone inhibiting the further breakdown of this substance, whilst in the presence of fluoride phosphoglyceric acid accumulated.¹

The change of hexose diphosphate to approximately equimolecular amounts of phosphoglyceraldehyde and phosphodihydroxyacetone was shown with *coli* juice acting on hexosediphosphate in the presence of bisulphite; the latter served to fix the two components of triosephosphate which were then estimated. In the absence of the fixative the enzyme isomerase immediately comes into action (as in the case of yeast juice) and an equilibrium is established with about 5% phosphoglyceraldehyde and 95% phosphodihydroxyacetone.²

It has also been shown that, as in the case of yeast juice, the change of phosphoglyceric to phosphopyruvic is reversible, the same equilibrium point being reached whether phosphoglyceric or phosphopyruvic is used as the starting-point, and that phosphopyruvate is transformed to pyruvate with the transfer of the phosphate to adenylic acid, A.T.P. being formed.³

So far the changes from hexose diphosphate to pyruvic acid are similar to those of yeast and muscle. The early stages between glucose or polysaccharide and hexosediphosphate await investigation.

Fig. 3 represents the scheme, the dotted lines and compounds in brackets representing unverified reactions and products.

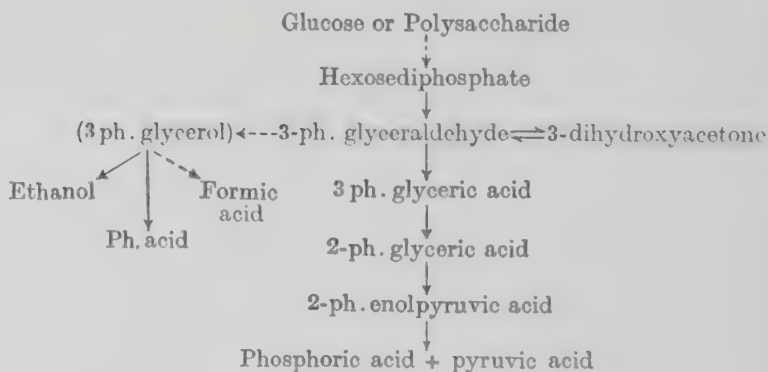


FIG. 3

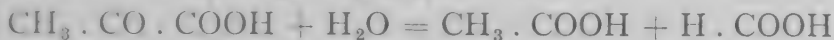
¹ Endo, 1938.

² Utter & Werkman, 1941.

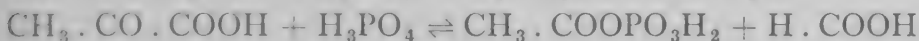
³ Ibid., 1942.

The breakdown of pyruvic acid

The decomposition of pyruvic acid by coliform organisms (and other bacteria so far tested) differs from that catalysed by yeast and muscle enzymes. No case of bacterial decarboxylation has so far been demonstrated (though cases of bacterial alcoholic fermentation where it might be expected have not yet been tested). In the case of *Esch. coli* and other organisms giving negative Voges Proskauer reactions pyruvic acid is decomposed into acetic acid and formic acid by a hydrolytic split. Thus:



This is in fact an example of a general reaction first shown by Neuberg in which α -keto acids are split into formic acid and a fatty acid having one carbon atom less than the α -keto acid.¹ By use of cell-free juice from *Esch. coli* evidence has been obtained that this reaction proceeds by way of a phosphoroclastic split



A cell-free preparation of cells decomposes pyruvate with formic and acetic as the principal products.²

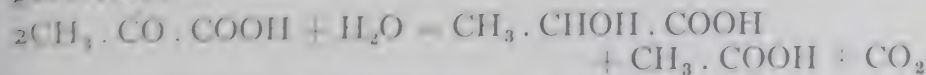
It was found by dialysing the enzyme preparation that phosphate, cocarboxylase, Mn (or Mg) are necessary to the functioning of the system. During the experiment acid-labile P. accumulates, probably attributable to acetylphosphate; in presence of A.A. this compound is missing but A.T.P. accumulates.

The reversibility of this reaction was shown by the use of C^{13} . HC^{13}OOH was prepared by incubating suspensions of *Esch. coli* with normal formic acid in an atmosphere of H_2 and C^{13}O_2 (Woods' reaction). The resulting HC^{13}OOH was incubated with pyruvic acid in the presence of the organism; when the fermentation of the pyruvic acid was still incomplete C^{13} was found in the carboxyl group.



In a similar way $\text{CH}_3\text{C}^{13}\text{OOH}$ was shown to condense with HCOOH to give $\text{CH}_3\text{C}^{13}\text{O} \cdot \text{COOH}$ in the presence of A.T.P.³

Both intact cells and cell-free extracts of *Esch. coli* produce lactic acid in small amounts from pyruvic,^{4, 5} in addition to acetic and formic. This probably arises by a dismutation as in the case of the gonococcus.⁶



since no reduction of pyruvate by H_2 (in presence of hydrogenase)

¹ Neuberg, 1914.

² Kalnitsky & Werkman, 1943.

³ Utter, Lipmann & Werkman, 1945.

⁴ Tilka, 1935.

⁵ Kalnitsky & Werkman, 1943.

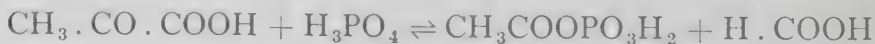
⁶ Krebs, 1937 (1).

or other donors (e.g. formate and formic dehydrogenase) can be demonstrated.¹

In organisms of the aerobacter group and others producing acetoin and 2:3-butylene glycol, pyruvic acid is decomposed by a third method as follows:



The partition of the available pyruvic acid between the hydrolytic (or phosphorolytic) enzyme and the acetoin enzyme depends on *pH*. In the first place the acetoin enzyme is almost absent from cells grown at *pH* 7.5 and over;² if grown in the presence of glucose both enzymes are present, but at *pH* 8.0 only the hydrolytic (or phosphorolytic) reaction occurs, whilst below *pH* 6.8 the acetoin enzyme only is active (*pH* optimum 3.5). The addition of $\text{CH}_3 \cdot \text{CHO}$ does not influence the production of acetoin by this organism.³ It was shown that acetic acid added to fermentations of glucose by *Aerobacter aerogenes* resulted in an almost quantitative increase in butylene glycol.⁴ $\text{CH}_3 \cdot \text{C}^{13}\text{OOH}$ added to a glucose fermentation by suspensions of *Aerobacter aerogenes* resulted in the formation of butylene glycol in which C^{13} was exclusively in the hydroxyl C. When $\text{C}^{13}\text{H}_3 \cdot \text{C}^{13}\text{OOH}$ was fed into the fermentation the C^{13} was distributed equally between the methyl and hydroxyl carbon atoms. Calculations indicated that in the former case about 7% and in the latter case about 13% of the butylene glycol was formed from acetic acid. These results were regarded as evidence that in the intact cells of *Aerobacter aerogenes* acetoin arises by the condensation of two 2-carbon compounds, possibly CH_3CHO or some derivative, but they are also explicable on the reversibility of the reaction



Similar evidence is provided by *Aerobacillus polymyxa*, where the addition of either acetaldehyde or acetate to fermenting cultures increased the yield of acetoin and butylene glycol. These experiments are in line with the classical work of Neuberg and Hirsch, who first showed the condensation of two mols. of acetaldehyde to acetoin in the case of yeast.⁵

The further anaerobic disruption of formic acid occurs through the enzyme formic hydrogenlyase. The reaction



was first studied by Pakes and Jollyman in 1901.⁶ These workers showed that gas production in a number of organisms (mostly of

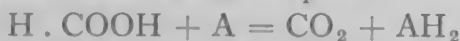
¹ Unpublished observations.

² Silverman & Werkman, 1941.

³ Ibid., 1940. ⁴ Reynolds *et al.*, 1937. ⁵ Neuberg & Hirsch, 1921.

⁶ Pakes & Jollyman, 1901.

the *Enterobacteriaceae*) studied by them was due to the decomposition of formate produced from hexose as an intermediate product. Meanwhile another enzyme decomposing formate had been found, viz. formic dehydrogenase catalysing the transfer of 2H from formate to oxygen or some other acceptor.¹

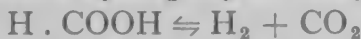


A third enzyme associated with this group is hydrogenase catalysing reductions by molecular hydrogen;² it has been obtained cell-free.^{3, 4}



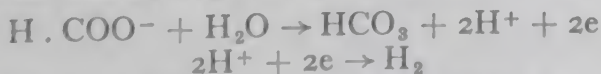
All these three enzymes are found in members of the *Enterobacteriaceae* and in many other groups, but do not seem to occur in organisms other than bacteria and a few *algæ*.

It is believed that the production of H₂ from formate is due to a distinct enzyme formic hydrogenlyase catalysing the reaction



This enzyme is partially adaptive and is practically absent from organisms grown on the surface of agar; it is present in organisms grown anaerobically and is increased by the presence of fermentable hexose or formate. The reversibility of the reaction was shown by Woods.⁵ The suggestion that formic hydrogenlyase is a combination of formic dehydrogenase and hydrogenase is rendered unlikely by the fact that a strain of *Bact. dispar* was found possessing both these enzymes but producing no gas from formate; in addition a strain of *Aerobacter aerogenes* was found which produced hydrogen but had no hydrogenase.⁶

Recent work by Waring and Werkman⁷ has, however, provided an alternative interpretation of results. It has been shown with *Aerobacter indologenes* grown on an iron-deficient medium that the three enzymes hydrogenase, formic dehydrogenase and formic hydrogenlyase are all absent or present in traces only. This suggests that these are all iron-containing enzymes. Furthermore formic hydrogenlyase was formed as freely in strongly aerated cultures as anaerobically. These workers believe that formic hydrogenlyase is really a combination of formic dehydrogenase and hydrogenase. They claim that there is no organism known which contains formic hydrogenlyase and does not also possess formic dehydrogenase and hydrogenase (see, however, the case of *dispar*



¹ Quastel & Whetham, 1925 (1).

² Beyerlinck, 1941.

³ Woods, 1936 (1).

⁴ Waring & Werkman, 1944.

² Stephenson & Stickland, 1931.

⁴ Back *et al.*, 1946.

⁶ Stephenson & Stickland, 1932.

above) and postulate the production of H_2 by the intervention of an iron-containing electron carrier.¹

At any rate the production of H_2 appears to be more sensitive to Fe deficiency than the other two enzymes implicated, which may be interpreted by regarding it as a separate enzyme or as depending on an electron-carrying system highly sensitive to Fe deficiency.

On the other hand, Kalnitsky and Werkman² report that aerating *Esch. coli* prevents the formation of the lyase, though formic dehydrogenase and hydrogenase are both present.

Our present knowledge of the anaerobic decomposition of pyruvic acid may be diagrammatically represented as in Fig. 4.

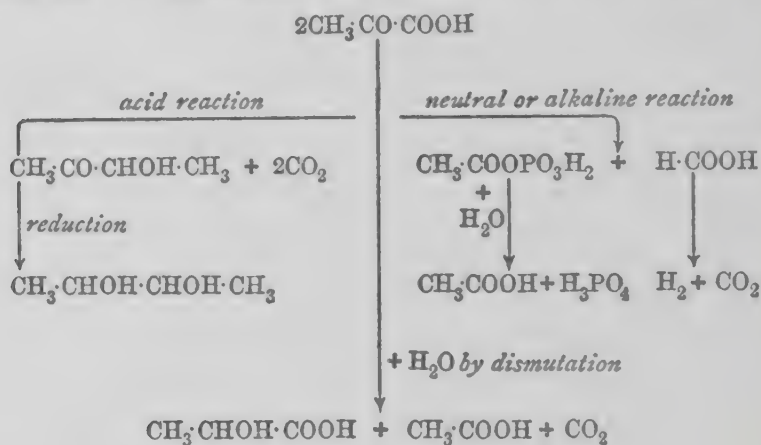


FIG. 4

Fermentation of glycerol

Glycerol was shown by Harden³ to be fermented by *B. coli*, ethyl alcohol and formic acid being the principal products. A detailed study of the same fermentation, using various members of the *Bacteriaceae*, was made by Braak⁴ on the same lines as the work of Scheffer with glucose. The products obtained were qualitatively the same as from glucose but the proportion of the oxidised products was lower and that of the reduced products higher. In the case of *B. lactis aerogenes*, however, acetoin and 2, 3-butylene glycol appear only in traces.

The fermentation of glycerol as carried out by Braak in peptone or yeast water is characterised by one important feature absent in the case of glucose. When the course of the fermentation is followed (by measurement of gas evolved) it is found to come to a standstill long before the glycerol is completely fermented; if now more peptone or yeast water is added the fermentation again starts and again stops short of completion. It was found that the

¹ Ordal & Halvorsen, 1939.

³ Harden, 1901.

² Kalnitsky & Werkman, 1943.

⁴ Braak, 1928.

added peptone could be replaced by substances such as aspartic acid or methylene blue acting as hydrogen acceptors. It is clear that in order for glycerol to be transformed to a compound of the $C_3H_6O_3$ group it must first lose hydrogen, and initially this must occur by means of an external hydrogen acceptor though later the intermediate fermentation products may take on the role. Fermentation of glycerol in a synthetic medium without such an oxidising agent does not occur. One member of this group of bacteria, however (*Bact. freundii*), fermented glycerol without the addition of a hydrogen acceptor and carried the fermentation through even in a purely mineral medium. The quantitative estimation of the products led to the disclosure of a deficit and it was eventually found that an unsuspected substance was present in considerable amounts. This turned out to be trimethyleneglycol, a reduction product of glycerol, $CH_2OH \cdot CH_2 \cdot CH_2OH$. It would thus appear that this organism is able to carry out a dismutation with two molecules of glycerol, thus dispensing with an external oxidising agent.

The same fermentation has been reported by certain strains of *B. lactis aerogenes* (*Aerobacter*);¹ here trimethylene glycol amounted to over 40% of the fermentation products.

A fermentation of glycerol in which succinate is the principal product has been recorded by Krebs,² 40.6 mols. succinic acid being obtained by the fermentation of 100 mols. of glycerol; the fermentation was carried out by a washed suspension of *Bact. coli* in bicarbonate buffer.

Fermentation of dihydroxyacetone

The products of the fermentation of dihydroxyacetone by *Bact. coli* are as follows:

Glycerol,	37-50%	of dihydroxyacetone fermented
Acetic acid,	20-22%	„ „
Formic acid,	4-15%	„ „

with some carbon dioxide and hydrogen and occasionally some succinic acid.

The fermentation is probably due to an adaptive enzyme. This is shown by the relative rates of fermentation of glucose and dihydroxyacetone by washed suspensions of bacteria grown with and without dihydroxyacetone in the medium.

L-Glyceric acid is decomposed by *Bact. coli* into acetic and formic acids and ethyl alcohol,³ and *L*-glyceraldehyde to the same products as dihydroxyacetone with the addition of lactic acid.⁴

¹ Maclellan & Werkman, 1940.

² Virtanen & Peltola, 1939.

³ Krebs, 1937 (2).

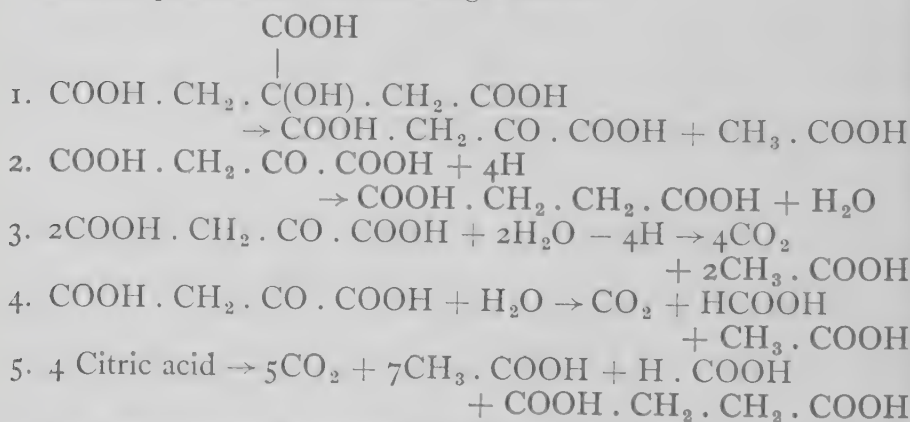
⁴ Virtanen & v. Hansen, 1932.

Fermentation of citric acid

This fermentation was first effectively studied by Deffner¹,² with an organism later identified as *Aerobacter aerogenes*. Brewer and Werkman³ demonstrated an identical fermentation due to a strain of the same organism.

Deffner showed that oxaloacetic acid and citric acid are fermented to the same end products, viz. ethanol, CO₂, formic, acetic and succinic acids. In the case of citric acid between three and four times as much acetic appeared as in the case of oxaloacetic. From Deffner's data the scheme is advanced that the key reaction in the fermentation of citric acid is the splitting off of acetic with the formation of oxaloacetic. The enzyme responsible for this reaction is adaptive and suffices to confer the ability to ferment citrate since the subsequent reactions by which oxaloacetic is transformed are common to the *enterobacteriaceæ* and to other organisms.

Deffner postulated the following scheme :



The experimental verification for this scheme is presented in Table 2.

TABLE 2

CO ₂ mols.	CH ₃ · COOH mols.	H · COOH mols.	COOH · CH ₂ · CH ₂ · COOH mols.
1 mol. citric 1·25 1·1-1·3	1·75 1·68-1·86	0·25 0·23-0·38	0·25 0·1-0·24
			cal. found

In addition (and not represented above) a small amount of ethanol is also formed.

The steps postulated do not represent distinct enzymic reactions but all can be accounted for as the result of enzymes known to occur in bacteria.

¹ Deffner, 1939.

² Deffner & Franke, 1939.

³ Brewer & Werkman, 1939.

A fermentation of citric acid by the heterofermentative lactic organism *Str. paracitrovorum*¹ yielded a series of similar products, whilst several homofermentative organisms yield CO₂ and acetic acid as the main products, with formic and lactic acids but no succinic.²

Propionic fermentation

This fermentation is brought about by a group of gram-positive rod-shaped organisms at present regarded as a family of the *Lactobacteriaceae*. The organisms can be readily obtained from the rumen or faeces of the cow or the sheep and occur also in Gruyère and Emmentaler cheese. The process was early investigated by Fitz,³ who showed that during the fermentation in question acetic and propionic acids were produced not only from glucose but also from malic and lactic acids. The fermentation of lactic acid is important as this compound is usually an end product of fermentation; here it appears to undergo an oxidoreduction or dismutation according to the quantitative investigations of Fitz:

$$3\text{CH}_3 \cdot \text{CHOH} \cdot \text{COOH} = 2\text{CH}_3 \cdot \text{CH}_2 \cdot \text{COOH} + \text{CH}_3 \cdot \text{COOH} + \text{CO}_2 + \text{H}_2\text{O}$$

this has been confirmed by others,⁴ with organisms isolated from Emmentaler cheese, the "eyes" of which are due to the CO₂ liberated.

Virtanen⁵ proceeded further with this study, showing that besides lactic acid, the following were all fermented: glucose, fructose, mannose and lactose, sucrose and xylose, the products and proportions being the same as in the case of lactic acid. Pyruvic was fermented as follows:



whilst glycerol fermented in yeast water gives rise to an almost quantitative yield of propionic acid.⁶

The origin of succinic acid

In this as in many other fermentations succinic acid frequently appears. Van Niel, who first studied this question, believed that the succinic acid formed was due to the deamination and reduction of aspartic acid present in the yeast water and hence was unrelated to the fermentation. This undoubtedly contributed to the succinic acid obtained in his experiments but, as later became apparent, was not the principal part of the story.

The solution of the problem of the origin of succinic acid in

¹ Hule & Werkman, 1941.

² Campbell & Gunsalus, 1944.

³ Fitz, 1872, 1879, 1880.

⁴ v. Freudenreich & Orla Jensen, 1906.

⁵ Virtanen, 1923.

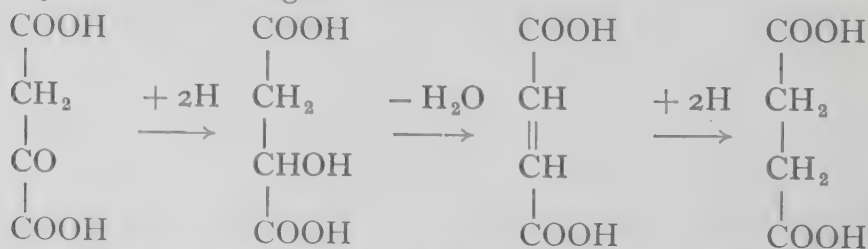
⁶ Van Niel, 1928.

fermentation generally was first solved in studies on propionic fermentation and for that reason is dealt with in this place ; but the reactions appear to be quite general for bacteria and indeed also for animal tissues.

It was first observed by Wood and Werkman that in a propionic fermentation of glycerol carried out in a closed system in an atmosphere of CO_2 the total carbon of the end products exceeded that of the carbohydrate fermented. This they attributed to fixation of CO_2 in the fermentation products. Furthermore they were able to show that at each stage in the fermentation the mols. CO_2 fixed and succinic acid produced were approximately equal.¹ The fixation of CO_2 and production of succinic acid are favoured by the presence of phosphate in the growth medium and inhibited by fluoride in the reaction vessel ; evidence was obtained that succinic acid was also formed by a mechanism insensitive to fluoride.²

The fixation of CO_2 in succinic acid was proved by the use of heavy carbon (C^{13}). The normal percentage of C^{13} in naturally occurring compounds is 1.09. A fermentation of glycerol was carried out in bicarbonate containing 4.64% of C^{13} . At the end of the experiment the amount of each product was estimated and also the percentage of C^{13} in it. Any significant increase in C^{13} above 1.09 indicates that C^{13} from the added bicarbonate had entered the molecule. The analysis showed that C^{13} was present in succinic, propionic and acetic acids and in propyl alcohol. The presence of C^{13} in the propionic acid (and alcohol) suggests that the propionic acid does not arise from glycerol by reduction but that it also passes through a 4-carbon stage.³ Similar experiments on the fermentation of galactose and of pyruvate by *Esch. coli* showed C^{13} in succinate and formate only.

It has been shown that various strains of propionic bacteria can carry out the following reactions :^{4, 5}



Hence if oxaloacetic acid is the first product of CO_2 assimilation it is easy to account for the presence of C^{13} in succinic acid in the experiments with the C^{13} isotope recorded above.

¹ Wood & Werkman, 1938.

² Ibid., 1940.

³ Wood, Werkman, Hemingway & Nier, 1941.

⁴ Krebs & Eggleston, 1941.

⁵ Nishina *et al.*, 1941.

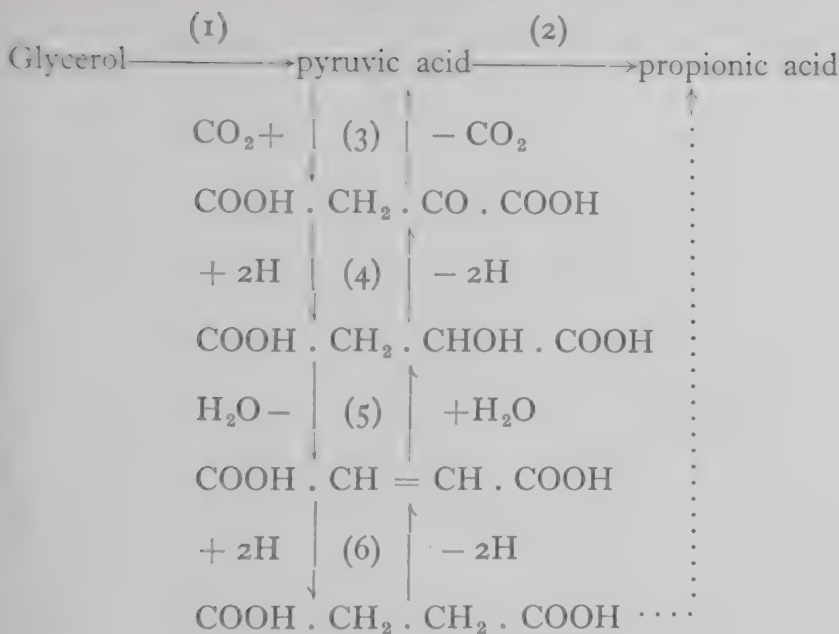


FIG. 5

The scheme shown in Fig. 5 depends on the occurrence of reaction (3) and on its reversibility. This involves the existence of an enzyme decarboxylating oxaloacetic at the carbon atom adjacent to the methylene group. Such an enzyme was first found in lysed cells of *Micrococcus lysodeikticus*¹ and later in cell-free preparations of *Esch. coli*.² In preparations containing Mg and Mn and freed from cocarboxylase the production of pyruvic and CO₂ from oxaloacetic in accordance with reaction (3) was proved. The demonstration of its reversibility was, however, difficult. This is due partly to the equilibrium being greatly in favour of the pyruvic and CO₂, and partly to the extreme reactivity of oxaloacetate. By careful adjustment of conditions, however, and in presence of cyanide, the production of oxaloacetate from pyruvate and CO₂ was finally achieved.³

The occurrence of C¹³ in the carboxyl of the propionic acid when glycerol is fermented in the presence of C¹³O₂ requires some explanation. In general the use of isotopes in fermentation chemistry has demonstrated the much greater interchange amongst products of fermentation (due to the reversibility of many enzyme actions) than was at first realised. The case in point is explicable on the known reversibility of the enzymes in Fig. 5.

The earlier unproven theory⁴ of the formation of succinic acid by the condensation of 2 mols. of acetic acid appears unnecessary

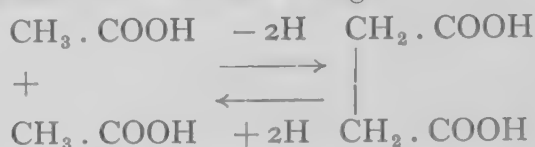
¹ Kramnitz & Werkman, 1941.

² Kalnitsky & Werkman, 1943 (2).

³ Ibid.

⁴ Harden, 1901.

in view of its production from pyruvic and CO_2 . However, evidence obtained by the use of heavy carbon shows that the former reaction also occurs. Using washed cell suspensions of *Aerobacter aerogenes*, $\text{CH}_3 \cdot \text{C}^{13}\text{OOH}$ was added to the fermentation of glucose. Significant amounts of succinic acid with C^{13} in the carboxyl were isolated. The addition of succinate containing C^{13} in the carboxyl resulted in the isolation of $\text{CH}_3\text{C}^{13}\text{OOH}$. This could not arise by the route succinic \rightarrow fumaric \rightarrow malic \rightarrow oxaloacetic \rightarrow pyruvic \rightarrow acetic + CO_2 because in that case the C^{13} would be found in C^{13}O_2 . It seems, therefore, that we are driven to postulate the reversible change

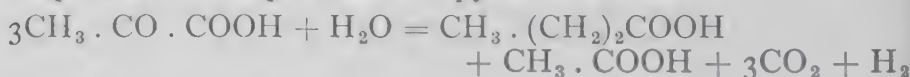


where the C^{13} is found exclusively in the carboxyl. The above scheme represents only the overall reaction and is not intended to indicate the 2- and 4-carbon compounds actually reacting. When $\text{C}^{13}\text{H}_3 \cdot \text{C}^{13}\text{OOH}$ was fed to the fermentation, succinic acid was isolated having C^{13} in both methylene and carboxyl carbon atoms.

Obviously the reaction postulated above requires an acceptor for 2H . In the fermentation under discussion this may be acetoin, which is readily reduced to butylene glycol.

Butyric and butyl fermentations

A large number of *Clostridia* effect fermentations in which butyric acid and related compounds predominate; *Cl. tetanomorphum*, for example, ferments pyruvic acid thus:¹



These fermenters have been classified as follows:²

1. Butyric fermenters producing mainly butyric and acetic acids CO_2 and H_2 . (Type *Cl. butyricum*.)
2. Butyl fermenters producing in addition ethanol, butanol and acetone. (Type *Cl. acetobutylicum*.)
3. That section of 2 in which acetone is reduced to isopropanol. (Type *Cl. butylicum*.)

Table 3 shows the products of fermentations in each of these groups. It is probable that the fermentation of group 1 represents the parent primitive type and suggests that the production of the alcohols, acetone and isopropanol appears as a result of additional enzymes acquired more recently in special conditions of nutrition.

¹ Woods & Clifton, 1937.

² McCoy *et al.*, 1930.

TABLE 3

FERMENTATION OF 2% GLUCOSE (IN YEAST WATER) BY GROWING CULTURES

Products	Percentage of glucose fermented		
	<i>Cl. butyricum</i> ¹	<i>Cl. acetobutylicum</i> ²	<i>Cl. butylicum</i> ³
CO ₂	46.9	54.0	54.9
H ₂	2.65	1.5	0.1
Formic acid	none	none	trace
Acetic acid	26.9	4.7	1.0
Butyric acid	27.9	2.1	0.9
Lactic acid	none	trace	none
Ethanol	"	2.4	2.9
Butanol	"	23.0	28.1
Acetone	"	7.2	0.2
Isopropanol	"	none	9.2
Acetoin	"	3.1	none

* Reported as *Granulobacter saccharobutyricum*.

Thus *Cl. acetobutylicum* when growing on maize meal produces its full complement of fermentation products, but if grown on a medium of salts, casein digest, glucose and asparagine or ammonium phosphate it produces no (or traces only of) butanol or acetone and reverts to an acid fermentation resembling that of *Cl. butyricum*.

The fermentation becomes complete with respect to the missing products when the medium is enriched with yeast autolysate, tryptic digest or autolysate of liver or tryptic digest of maize meal. These addenda all work at an optimum concentration above and below which their effectiveness falls off till it reaches zero. It is also necessary to replace ammonium phosphate by asparagine.^{3, 4} The complete medium equivalent to maize meal is found in Appendix (Medium III).

Fig. 6 shows (in agreement with previous observations on this and related organisms) that during the early stages the fermentation is an acid one and that the secondary products (acetone and butanol) occur only after the pH has fallen to about 6.0. Moreover it has been shown that if the fermentation is kept neutral by the addition of CaCO₃ or NaHCO₂ no solvents are formed.^{5, 6} Simultaneously with the appearance of butanol and acetone, butyric and acetic acids decrease, indicating that they are the precursors of butanol and acetone. This is substantiated by the addition of these compounds to an active fermentation of glucose when the yields of butanol and acetone rise correspondingly.⁷ Aceto-

¹ Donker, 1926. ² Van der Lek, 1930. ³ Davies & Stephenson, 1941.⁴ Tatum *et al.*, 1935. ⁵ Bernhauer *et al.*, 1936. ⁶ Osburn *et al.*, 1937.⁷ Ibid., 1938.

acetic acid added to a fermentation appears as acetone.¹ Pyruvic acid is fermented by these organisms mainly to acetic acid, acetone and acetoin,² or to acetic acid, butyric acid and acetone;³

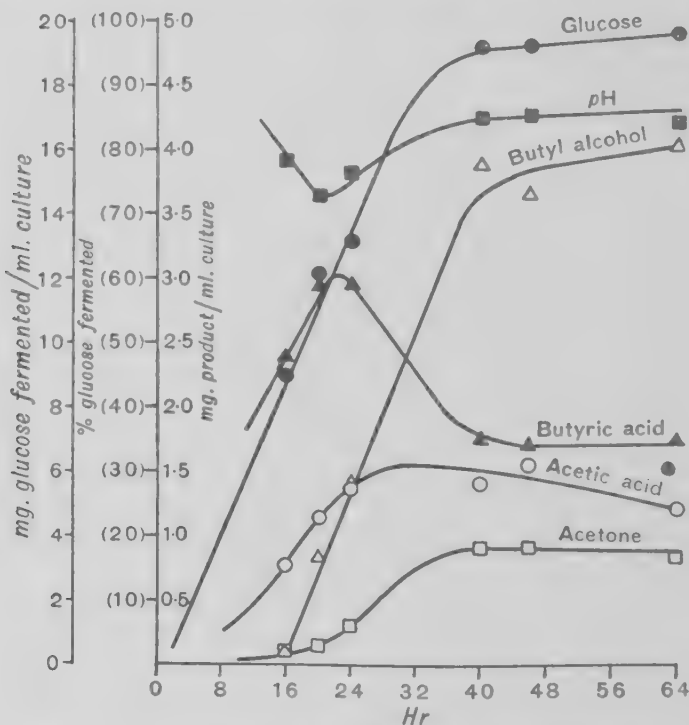
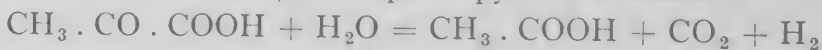


FIG. 6.—Fermentation of glucose by *Cl. acetobutylicum*—course of reaction⁴

a cell-free preparation of *Cl. butylicum* (obtained by extracting frozen cells with water) decomposed pyruvic acid thus :⁵



The reaction was shown to be improved by liver extract and dependent on phosphate and is probably a phosphorolysis.

Evidence from the use of washed suspensions

The use of washed suspensions was for long rendered impossible in the case of *Cl. acetobutylicum* by the rapid falling off in enzyme activity after centrifuging and suspending in buffer. This was eventually surmounted by using as the suspending fluid⁶ the complete growth medium from which glucose had been omitted. Cells prepared in this way showed a curious qualitative variation in fermentative ability ; those harvested early in the growth period

¹ Johnson *et al.*, 1933.

² Ibid.

³ Davies, 1942.

⁴ Davies & Stephenson, 1941, *Biochem. J.*, **35**.

⁵ Koepsell & Johnson, 1942.

⁶ Davies & Stephenson, 1941.

fermented rapidly but produced no solvents, whilst those harvested after the appearance of acetone in the growth medium produced a complete fermentation (see Table 4).

TABLE 4¹

EFFECT OF AGE OF CULTURE ON ACETONE PRODUCED (a) IN THE CULTURE MEDIUM, (b) BY CELL SUSPENSIONS PREPARED FROM IT

Age of culture hr.	• Growth mg./ml. dry wt.	Percentage of glucose fermented	Acetone in culture mg./ml.	Acetone formed by cell suspensions mg./ml.
6	0.291	5.0	none	none
8	0.965	11.8	none	none
10	1.300	25.3	0.038	0.090
12	1.340	33.9	0.090	0.154
14	1.300	38.0	0.123	0.190
17	1.240	40.2	0.163	0.111
22	1.350	41.6	0.181	0.091
24	1.085	44.8	0.193	0.251
43	0.695	71.5	0.707	0.082

The mechanism by which butyric acid is reduced to butanol in the latter part of the fermentation is not very clear. The fact that this occurs only at acid reactions indicates that the undissociated acid forms the reductant. The reducing agent is not certain. This organism possesses no dehydrogenases for succinic or formic acids or for ethanol, but it has a powerful hydrogenase, and dehydrogenases for glucose and pyruvic acids. Hydrogen, however, does not reduce butyrate in the presence of the organism.² Indirect evidence obtained by adding fresh glucose to a fermentation after about half the original glucose had disappeared and comparing the butanol produced with that of the uninterrupted fermentation indicates that glucose—or possibly triosephosphate—serves as the hydrogen donator for this reduction. No evidence was obtained that pyruvate functions in this way with *Cl. acetobutylicum*,³ though in the case of *Cl. butylicum* it has been shown that pyruvic acid can reduce butyric acid at pH 4.8 but not at 6.2.⁴

The immediate precursor of acetone is certainly acetoacetic acid.⁵ *Cl. acetobutylicum* grown on the complete medium for acetone production rapidly decarboxylates acetoacetic acid. From acetone-treated cells an enzyme acetoacetic acid decarboxylase has been extracted by water in the presence of caprylic alcohol; the enzyme was purified by adsorption on to alumina C γ , elution and fractional precipitation by (NH₄)₂SO₄ and ethanol. The final product had a Q_{co_2} of about 4×10^5 . The enzyme tends to dissociate at low concentrations into enzyme and coenzyme. The latter

¹ Davies & Stephenson, 1941.

² Davies, 1942.

³ Ibid.

⁴ Brown *et al.*, 1937.

⁵ Johnson *et al.*, 1933.

has not been identified but is related to, though not identical with, diaphorase; evidence points to its possible identity with riboflavin phosphate.¹

The origin of acetoacetic acid from acetic is obscure. Acetic acid added either to a glucose or to a pyruvate fermentation greatly increases the yield of acetone^{2, 3, 4} (see Fig. 7).

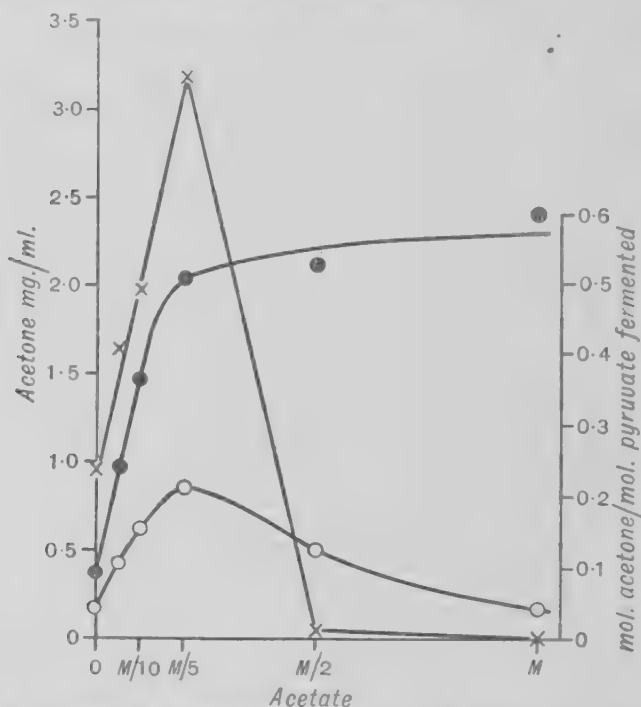


FIG. 7.—Effect of acetate concentration on acetone production by *Cl. acetobutylicum* (Weizmann). The reactions were carried out in Krebs vessels containing 2 ml. *M* glucose or 1 ml. 0.6 *M* pyruvic acid, 5 ml. cell suspension (15 mg. dry wt./ml.) and sufficient 2*M* acetate buffer pH 5.0 to give final acetate concentrations of nil, *M*/20, *M*/10, *M*/5, *M*/2, and *M*; the total volume was made up to 20 ml. with water. Gas phase H_2 ; temp. 38°. Incubated for 18 hrs. and then analysed for acetone. ○—○ Pyruvate as substrate (acetone mg./ml.). ●—● Pyruvate as substrate (acetone mols./mol. pyruvate fermented). x—x glucose as substrate (acetone mg./ml.)⁵

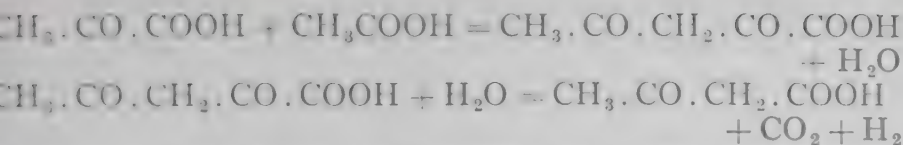
Acetate added to cell suspensions does not give rise to acetone so it is probable that the simultaneous fermentation of hexose or pyruvate is needed to provide the necessary energy.

The possibility that acetoacetic acid might arise from the condensation of acetate with pyruvate to form acetopyruvate has been tested with negative results.

¹ Davies, 1943. ² Reilly *et al.*, 1920. ³ Bernhauer & Kurschner, 1935.

⁴ Davies, 1942.

⁵ Davies, "Studies on the Acetone-Butyl Alcohol Fermentation," *Biochem. J.* (1942), 36, 592.



Acetopyruvic acid was added to actively fermenting glucose and maize meal. In neither case was it fermented but in both cases it inhibited the carbohydrate fermentation.¹

The uncertainty concerning the main problem in these fermentations, viz. the origin of the 4-carbon compound butyric acid, obviously called for the application of a fresh technique. This has been supplied by the use of heavy carbon compounds. Fermentations of maize mash by *Cl. acetobutylicum* and *Cl. butylicum* have been carried out in the presence of added intermediates with one or more labelled carbon atoms. The end products of the fermentation with the percentage of C¹³ in each were estimated by the usual methods. The results of this important investigation are recorded in Tables 5, 6 and 7. The percentage excess of the C¹³ and the percentage recovery of the total added C¹³ in each fermentation product together supply the relevant data on which deductions from the experiment are based.²

TABLE 5³

DISTRIBUTION OF C¹³ OF CH₃·C¹³OOH AMONG THE PRODUCTS OF FERMENTATION BY THE BUTYL ALCOHOL BACTERIA

Medium: 100 ml., 3.75 g. maize meal, 5.77 mM K₂HPO₄, 6.15 mM CH₃·C¹³OOH; in addition 0.1 g. Bacto yeast for *Cl. butylicum*.

	<i>Cl. acetobutylicum</i>				<i>Cl. butylicum</i>			
		C ¹³ in excess of normal	C ¹³ in excess of normal	Recovery of C ¹³ of added acetic acid		C ¹³ in excess of normal	C ¹³ in excess of normal	Recovery of C ¹³ of added acetic acid
	mM	%	mM	%	mM	%	mM	%
acetone	5.04	0.72	0.109	15.0	—	—	—	—
propanol	—	—	—	—	5.38	0.84	0.136	19.0
ethanol	8.19	1.25	0.410	55.0	6.37	1.20	0.308	43.0
methanol	1.89	0.54	0.020	3.0	0.23	0.70	0.003	0.4
acetic acid	4.40	0.43	0.038	5.0	1.06	0.24	0.005	1.0
butyric acid	0.75	0.70	0.021	3.0	3.36	0.17	0.023	3.0
formic acid	0.36	0.14	0.002	0.3	0.10	0.10	0.000	0.0
CO ₂	35.40	0.38	0.135	18.0	35.00	0.53	0.186	26.0
lignin	15.60*	0.80	0.013	2.0	34.40*	0.05	0.017	2.0
alcohol	15.10*	0.05	0.008	1.0	—	—	—	—
		Totals	0.756	101.0		Totals	0.678	94.0
acetic acid added	6.15	6.99	0.749	—	6.15	5.87	0.722	—

* M.M.C. in fraction from fermented medium following removal of volatile compounds.

¹ Davies, 1942.

² Wood *et al.*, 1945.

³ *Ibid.*

FERMENTATION

TABLE 6¹

DISTRIBUTION OF C¹³ OF CH₃.C¹³H₂.CH₂.C¹³OOH AMONG THE PRODUCTS OF FERMENTATION BY THE BUTYL ALCOHOL BACTERIA

Medium: 45 ml., 1.70 g. maize meal, 2.15 mM KH₂PO₄, 2.43 mM CH₃.C¹³H₂.CH₂.C¹³OOHNa; in addition 0.05 g. Bacto yeast extract for *Cl. acetobutylicum*.

	<i>Cl. acetobutylicum</i>				<i>Cl. butylicum</i>			
		C ¹³ in excess of normal	C ¹³ in excess of normal	Recovery of C ¹³ of added butyric acid		C ¹³ in excess of normal	C ¹³ in excess of normal	Recovery of C ¹³ of added butyric acid
	mM	%	mM	%	mM	%	mM	%
Acetone .	1.47	0.04	0.002	2	—	—	—	—
Isopropanol.	—	—	—	—	1.71	0.08	0.004	3
Butanol .	4.98	0.51	0.102	84	4.70	0.53	0.100	83
Ethanol .	0.68	0.04	0.001	1	0.19	—	—	—
Acetic acid .	2.65	0.02	—	—	1.29	0.06	0.002	2
Butyric acid	1.03	0.38	0.016	13	1.47	0.07	0.004	3
CO ₂ .	16.50	0.01	—	—	14.90	0.01	—	—
Filtrate .	17.20*	0.02	—	—	19.98*	-0.01	—	—
Solids .	17.00*	0.00	—	—	22.00*	-0.01	—	—
		Totals	0.120	100		Totals	0.110	91
Butyric acid added .	2.43	1.24	0.121	—	2.43	1.24	0.121	—

TABLE 7²

DISTRIBUTION OF C¹³ OF CH₃.C¹³O.CH₃ AMONG THE PRODUCTS OF FERMENTATION BY THE BUTYL ALCOHOL BACTERIA

Medium: 60 ml., 2.25 g. maize meal, 1.69 mM of CH₃.C¹³O.CH₃; in addition 0.07 g. Bacto yeast extract for *Cl. acetobutylicum*.

	<i>Cl. acetobutylicum</i>				<i>Cl. butylicum</i>			
		C ¹³ in excess of normal	C ¹³ in excess of normal	Recovery of C ¹³ of added acetone		C ¹³ in excess of normal	C ¹³ in excess of normal	Recovery of C ¹³ of added acetone
	mM	%	mM	%	mM	%	mM	%
Acetone .	3.72	0.26	0.029	81	0.31	0.23	0.002	5
Isopropanol.	—	—	—	—	3.85	0.28	0.032	89
Butanol .	4.48	0.01	—	—	5.21	0.01	—	—
Ethanol .	1.05		—	—	0.11		—	—
Acetic acid .	1.49		—	—	0.40		—	—
Butyric acid	0.26	0.01	—	—	0.18	0.02	—	—
CO ₂ .	21.20	0.02	—	—	18.20	—	—	—
Filtrate .	27.70*	0.00	—	—	30.30*	-0.01	—	—
Solids .	30.60*	0.00	—	—	20.70*	-0.01	—	—
		Totals	0.029	81		Totals	0.034	94
Acetone added .	1.69	0.72	0.036	—	1.69	0.72	0.036	—

* mM of C in fraction from fermented medium following removal of volatile compounds

¹ Wood *et al.*, 1945.

² Ibid.

In Table 5 is shown the distribution of the C^{13} of the acetic acid added to the fermentation. In both fermentations about half the C^{13} was found in the butanol. This immediately disproves the idea that added acetate is quantitatively converted to acetone or isopropanol. However, these products contained a substantial fraction (15% and 19% respectively) of the C^{13} , and the CO_2 contained in each case slightly though not substantially more (18% and 26% respectively). This is in agreement with the production of acetone by the condensation of 2 mols. of acetic acid to acetoacetic acid and its subsequent decarboxylation. The acetic acid itself contained only 5% and 1% respectively of the C^{13} of the acetate added, showing that the greater proportion of the latter was built into other compounds and its place taken by acetic acid from the fermented starch. The high content of C^{13} in butanol as compared with butyric acid is explicable on similar lines; the butyric acid first formed would have contained the greatest content of C^{13} and become continuously diluted with butyric acid formed from fermentation acetic. Some C^{13} appeared in the ethanol, showing that this is derived from acetic acid, but less than in the case of butanol. This may indicate a dual origin of ethanol or formation at a later stage of the fermentation.

The experiment with added $CH_3 \cdot C^{13}H_2 \cdot CH_2 \cdot C^{13}OOH$ showed that 84% and 83% respectively was reduced to butanol. The small amount found in acetone and ethanol may be due to the reversal of the reactions leading to butanol synthesis. The added $CH_3 \cdot CO^{13} \cdot CH_3$ appeared as such mixed with fermentation acetone in the case of *Cl. acetobutylicum* and as isopropanol with *Cl. butylicum*; this is in accordance with previous evidence.

These experiments confirm previous work by other methods but supply in addition strong evidence that butyric acid arises from acetic through an unknown intermediate. The identity of this intermediate is still unknown. The possibility that acetoacetic acid may, in the early stages of the fermentation, be reduced instead of decarboxylated has been tested with negative results.¹

Lactic fermentation

Lactic acid forms an important unit among the products of many fermentations; it is, moreover, the only product of the fermentation of muscle. It is also noteworthy that many organisms producing mixed fermentations will, under conditions in which some of their enzymes are prevented either from being formed or from acting, revert to a fermentation in which lactic acid is the main product. Such, for example, are *Cl. butylicum* in the presence of CO_2 , *Cl. acetobutylicum* grown in a medium deficient in certain

¹ Davies, 1942.

¹ Kubowitz, 1934.

constituents or maintained at an alkaline pH ,¹ *Cl. welchii* grown in suboptimal concentrations of iron² or in the presence of carbon monoxide.

We may, perhaps, therefore provisionally regard the fermentation of hexose to lactic acid as the primitive basal form of fermentation from which other fermentations have arisen by the development of subsidiary enzymes. There exist, however, a number of organisms which produce lactic acid as the main or sole fermentation product; these are most of the gram-positive cocci and a group, also gram-positive, of lactic bacilli. Where lactic acid is the sole product the organisms are known as homofermenters; where small quantities of other products also occur the organisms are known as heterofermenters. It is now becoming increasingly apparent that the distinction between these latter is not very clear and that many so-called homofermenters may in certain conditions change over to heterofermentation. As, however, certain homofermentative organisms have clearly defined characters it may be well to consider some of these.

Among *Lactobacilli* derived from milk are groups distinguished by their respiratory systems.

The homofermentative lactic acid bacteria

A representative example of this group is *L. delbrückii*.³ This organism is distinguished by the absence of all hæmatin pigments and hence of cytochrome and cytochrome oxidase and catalase. It has, however, considerable amounts of a flavoprotein enzyme, in virtue of which it can oxidise glucose and lactate aerobically. Glucose is oxidised with production of H_2O_2 , which rapidly poisons the organism, causing the oxidation to fall off.



The addition of catalase to the system protects the enzyme from deterioration and allows the oxidation to proceed. Lactate and pyruvate are also oxidised with production of H_2O_2 , these oxidations being less sensitive to the poison than the glucose oxidation. —CN and CO do not appreciably inhibit oxidations by this organism.⁴

An interesting variant on this type of metabolism is *L. casei*; like *L. delbrückii* it lacks hæmatin pigments and has no catalase, but it appears to have no oxidising system at all. Hence no H_2O_2 is formed; oxygen is not toxic but behaves as an indifferent gas. These characteristics were established for two strains—whether they are true of all is uncertain.⁵ A third type of lactic organism

¹ Davies & Stephenson, 1941.

² Pappenheimer & Shaskan, 1944.

³ Meyerhof & Finkle, 1925.

⁴ Davies, 1933 (1, 2).

⁵ Ibid., 1933 (2).

was reported under the name of *Bact. cereale* and seems to have a different type of respiration, as it has a respiratory system inhibited by $-\text{CN}$ and CO and does not appear to produce H_2O_2 . It would seem, therefore, to possess hæmatin enzymes and probably catalase.¹

Change from homo- to heterofermentation

It has been shown in the case of growing cultures of *Streptococcus liquefaciens* that a change in the balance of fermentation products can be induced by growing at different values of pH.² Table 8 shows that the change from pH 5.0 to 9.0 alters the character of the fermentation from one which is predominantly lactic to the mixed type.

Many lactic organisms have been shown to ferment citric acid and use the energy thus liberated for growth in place of that derived from carbohydrate.³

TABLE 8

EFFECT OF pH ON THE PRODUCTS OF THE FERMENTATION OF GLUCOSE BY
Str. liquefaciens 815

Medium 1% tryptone, 0.2% yeast extract, 0.1% K_2HPO_4 and 1% glucose.
Experiments carried out at pH indicated. Products in mM of C_3 (glucose
× 2). Conditions semi-aerobic.

mM per 100 mM of C_3 (glucose) fermented	pH 5.0	pH 7.0	pH 9.0
Lactic acid	87.0	73.0	61.0
Acetic acid	6.1	9.4	16.6
Ethanol	3.5	7.3	11.2
Formic acid	7.7	16.8	26.4
Carbon recovered, % .	95.0	90.0	88.0

The co-ordination of fermentation and aerobic oxidation of carbohydrate : the "Pasteur Effect"

It was characteristic of Pasteur's genius to give a somewhat intuitive interpretation of his experiments and so to postulate the existence of phenomena of great importance which his actual experiments and the technical equipment of his time were insufficient to prove conclusively. An example of this faculty is his discovery that alcoholic fermentation is diminished in the presence of air, a phenomenon now known as the Pasteur Effect. Pasteur's quantitative experiments on the fermentation of sugar by yeast led him to the view that the main factor controlling fermentation was oxygen supply. In his first experiments,⁴ sugar solution with a small amount of protein (the latter as food for the growing yeast)

¹ Dancer, 1911 (1).

² Campbell & Gunsalus, 1944.

³ Ibid.

⁴ Pasteur, 1861 (1).

was placed in a flask from which air was expelled by boiling and which was then closed by a mercury seal and sown with a particle of yeast. A similar solution was exposed to the air in a thin layer at the bottom of a flask and likewise sown with yeast. In the comparatively anaerobic conditions of the first experiment the growth of the yeast was slow, but the breakdown of the sugar was rapid; in the aerobic conditions of the second experiment the converse was the case. A quantitative comparison of the yeast formed and sugar broken down in the two cases showed that for the production of 1 part of yeast 4 to 10 parts of sugar sufficed in aerobic conditions, whilst 60 to 80 parts were needed in anaerobic conditions. Pasteur regarded the excessive breakdown of sugar (i.e. "fermentation") as a direct result of the absence of free oxygen. He says:

The little cell known as brewers' yeast can develop by assimilating free oxygen so actively that this may be said to be its normal mode of existence, and, in so doing, it loses its fermentative character. . . . Since brewers' yeast assimilates oxygen so actively in the free state it proves that the cell requires it in order to live, and that, therefore, if this element cannot be obtained in the free state, it must be taken from the fermentable material; the cell ("plante") then appears to us as an agent for the breakdown of sugar.¹

Later experiments confirmed Pasteur in his views, and led him to enlarge his theory. The following data (Table 9)² may be taken as examples of the experiments on which he based his conclusions:

TABLE 9
EFFECT OF OXYGEN SUPPLY ON RATIO YEAST FORMED/SUGAR CONSUMED

Exp.	Conditions	Conc. of sugar, %	Vol. of sol., cc.	Initial wt. of sugar, gm.	Wt. of sugar used, gm.	Wt. of yeast formed, gm.	Yeast formed/Sugar used
1.	Very aerobic, 3 days	5.0	200	10.0	10.0	0.44	0.044
2.	Moderately aerobic, 9 days	5.0	3000	150.0	150.0	1.970	0.013
3.	Anaerobic, 19 days	5.0	3000	150.0	145.5	1.368	0.0094
4.	Very anaerobic, 3 months	5.0	3000	150.0	45.0	0.255	0.0056
5.	Very aerobic, 2 days	0.86	200	1.72	1.04	0.127	0.122
6.	Same, 24 hours	0.86	200	1.72	0.098	0.024	0.24

The theory of fermentation to which Pasteur was led by these and similar experiments is best conveyed by a translation of his own words:³

¹ Pasteur, 1861 (1).

² Ibid., *Études sur la Bière*, chap. vi.

³ Ibid., p. 251.

The inferences to be drawn from the preceding facts must be clear to everybody. For my own part I cannot help seeing in them the basis of the actual theory of fermentation. In the experiments that I have just described fermentation by yeast . . . appears as the direct result of the work of nutrition and of assimilation, that is of life, carried on in the absence of free oxygen. The heat¹ used in this work must necessarily be derived from the decomposition of fermentable material, that is from the sugars, which, like explosive substances, liberate heat on decomposition. Fermentation by yeast, therefore, seems to be closely related to the power possessed by this little cell of respiring in some way by means of the oxygen combined in the sugar. Its fermenting power (which must not be confused with fermenting activity or with the intensity of decomposition in a given time) varies considerably between the two limits fixed by the highest and lowest degrees in which it is possible for free oxygen to participate in the nutrition of the plant. If it is supplied with sufficient free oxygen for its life, nutrition, and respiratory combustion—in other words, if it is made to live like the ordinary moulds—it ceases to be a ferment, that is to say, the ratio between the weight of the plant and the weight of the sugar, which is its main source of carbonaceous food, is of the same order as in the case of the moulds. If on the contrary the yeast is deprived of the influence of air and made to develop in a sugar medium in the absence of free oxygen, it multiplies therein as if air were present, though less actively, and it is then that its fermentative character is most exaggerated and that the greatest difference exists, other things being equal, between the weight of yeast formed and the weight of sugar decomposed. In fact, if free oxygen participates in varying quantities, one can cause the fermenting power of the yeast to pass through all the stages between the extreme limits which we have just indicated. It seems to me that it could not be better established that fermentation is in direct relation to life when the latter is carried on without free oxygen, or in amounts of this gas which are insufficient for all the processes of nutrition and assimilation.

In examining closely this highly teleological interpretation of the phenomena of yeast fermentation, it must be admitted that the experimental evidence was insufficient to support the structure built upon it. For example, it is clear that Pasteur failed to distinguish between the requirements of the cell for oxygen *as such* and for energy. The proportion of oxygen in sugar is actually higher than in the average yeast cell, so no excessive breakdown of the former is necessary to supply the oxygen for the construction of the latter. The absence of free oxygen, however, deprives the cell of its readiest means of obtaining energy by the complete combustion of its organic substrate, and drives it to the more expensive method of incomplete combustion and anaerobic breakdown. Fermentative processes therefore serve the cell as a means for obtaining energy rather than oxygen. Pasteur regarded oxida-

¹ *Chaleur*, which should perhaps be translated *energy*.

tion and fermentation as alternative modes of life, the degree to which the one predominates over the other being governed by the oxygen supply—strict anaerobiosis resulting in pure fermentation, free aeration leading to complete oxidation. Pasteur's own experiments did not provide conclusive evidence for this view which has, however, been supplemented by later work. It is now known as the Pasteur Effect, the modern definition of which is the inhibition of fermentative breakdown caused by the utilisation of oxygen or respiration. The pertinent values in the case of yeast are as follows:

Q_{O_2} (buffer) measuring endogenous respiration and indicating oxidative capacity.

Q_{O_2} (glucose) measuring oxidation of glucose (+ endogenous respiration).

$Q_{CO_2}^{N_2}$ (glucose) measuring anaerobic fermentation.

$Q_{CO_2}^{air}$ (glucose) measuring fermentation in air.

Now if the result of carrying out a fermentation aerobically were to cause the oxidation of the anaerobic product of fermentation, e.g. alcohol or lactic acid, to completion, without any other effect on fermentation, the $Q_{CO_2}^{air}$ would be greater than the $Q_{CO_2}^{N_2}$ owing to the additional CO_2 produced by the reaction



Actually, however, in all types of yeast the $Q_{CO_2}^{N_2}$ is greater than the $Q_{CO_2}^{air}$, showing that oxygen actually represses fermentation; a similar phenomenon is observable in lactic fermentation. A measure of this repression is given by the formula

$$\frac{\left(Q_{CO_2}^{N_2} - Q_{CO_2}^{air} \right) 3}{Q_{O_2}}$$

which represents the number of glucose molecules inhibited from fermentation per mol. O_2 taken up; this is known as the Meyerhof Quotient.

From Table 10 it is seen that the more naturally aerobic types of yeast have a relatively high Q_{O_2} and correspondingly low Meyerhof Quotient, and that they tend to turn over more completely from fermentation to respiration in response to oxygen supply.

It has been pointed out by Meyerhof¹ that 1 atom of oxygen inhibits the formation of 1 molecule of ethanol; but if each molecule of ethanol were oxidised to completion 6 atoms of oxygen would be required instead of 1. The suggestion is therefore

¹ Meyerhof, 1942.

TABLE 10¹
MEYERHOF QUOTIENTS

Organism	Q _{O₂}	Q _{CO₂} ^{air}	Q _{CO₂} ^{N₂}	Meyerhof Quotient $(Q_{CO_2}^{N_2} - Q_{CO_2}^{air})_3$	Inhibition %
				Q _{O₂}	
Wild yeast . . .	— 180	18	260	4.0	93
Bakers' yeast . . .	— 87	95	274	6.2	65
Brewers' yeast . . .	— 8	214	233	7.5	8
<i>L. cereale</i> . . .	— 189	49	305	3.9	84
<i>L. delbrückii</i> . . .	— 190	79	188	3.0	58
<i>L. casei</i> . . .	0	287	316	—	9
<i>Prop. pentoseaceum</i> . . .	— 15	4	20	3.0	80

that when oxygen is admitted into the fermentation acetaldehyde (or pyruvic acid) is not reduced by dihydrocozymase but instead oxygen is reduced to water. Consequently the ratio between atoms of oxygen consumed and molecules of ethanol suppressed is 1 : 1. This explanation, however, sheds no light on the fate of the acetaldehyde or pyruvic acid, which remains unreduced. It is certain that they do not accumulate as such and if they disappear by oxidation the 1 : 1 ratio of atoms of oxygen consumed to mols. of ethanol suppressed would no longer hold. Both aerobically and anaerobically dihydrocozymase is oxidised to cozymase; the latter is again reduced by the oxidation of phosphoglyceraldehyde to phosphoglyceric acid (reaction 8), a reaction catalysed by triosephosphate dehydrogenase, but other oxidoreductions in the fermentation cycle could also play this part. Another way in which oxygen may bring about the Pasteur Effect is by inhibiting one or more of the enzymes of the glycolytic cycle. It has been shown, for example, that triosephosphate dehydrogenase is inhibited by the presence of oxygen and reactivated by —SH.²

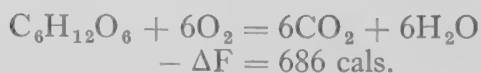
It has also been shown that certain redox indicators inhibit reaction 4 leading to the formation of hexose diphosphate.³ Following this it was shown by Engelhart and Sakov⁴ that the fermentation of hexosediphosphate was almost unaffected by redox indicators, e.g. indophenolsulphonate 0.125 *M*, whilst the fermentation of glucose was seriously inhibited. This suggested that the point where oxidation inhibits fermentation is prior to reaction 5. It was shown that reactions 1, 2 and 3 were also insensitive to oxidation and finally that the sensitive enzyme was 4, i.e. phosphohexokinase which phosphorylates the Neuberger ester, fructose monophosphate, to the fructose diphosphate. The inhibition first

¹ Lipmann, 1942.² Michaelis & Snythe, 1936.³ Rapkine, 1938.⁴ Engelhardt & Sakov, 1943.

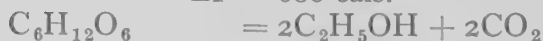
demonstrated with redox indicators was shown to occur with other oxidising agents such as iodine, quinone and dehydroascorbic acid; more important was the fact that cytochrome plus cytochrome oxidase completely inhibited the transphosphorylation process. Any such inhibition would be expected to lead to the accumulation of some previously occurring intermediate; no example of this seems to have been reported.

The demonstration by Engelhardt and Sakov that the inhibition of phosphohexokinase can be brought about by cytochrome and cytochrome oxidase seems to render the postulation of a special Pasteur enzyme unnecessary,¹ but there must clearly be alternative oxidising mechanisms in operation since *L. delbrückii* displays the Pasteur Effect whilst containing no hæmatin enzymes. Probably the flavine enzymes here effect the oxidation. A contributory factor may well be the effect of oxygen in oxidising the thiol compounds of the cell, whose function, whilst in the reduced state, seems to be to protect systems inactivated by oxidation.^{2, 3, 4}

The energy relations of the change from fermentation to oxidation has been discussed by Lipmann,⁵ who gives the following figures:



$$- \Delta F = 686 \text{ cal.}$$



or



$$- \Delta F = 54 \text{ cal.}$$

$$- \Delta H = 36 \text{ cal.}$$



$$- \Delta F/3 = 79 \text{ cal.}$$

$$- \Delta H/3 = 61 \text{ cal.}$$

Thus in the case of lactic and alcoholic fermentations 7.9% of the total free energy made available by oxidation is obtained by fermentation, whilst in propionic fermentation, owing to the dismutation occurring between lactic acid molecules, the yield is 11.5%. So in anaerobic conditions the cell adopts a relatively wasteful system of breakdown which is immediately inhibited when oxygen becomes available. The more complete this turn-over the more efficient the growth processes of the organism.

¹ Stern & Melnick, 1941.

³ Rapkine, 1938.

⁵ Lipmann, 1942.

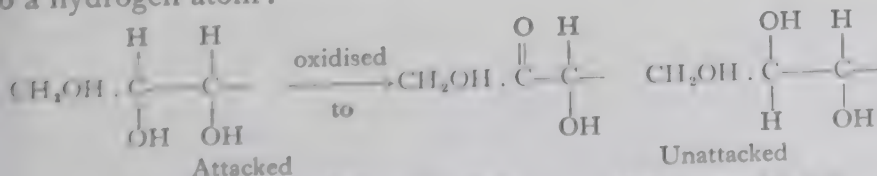
² Quastel & Wheatley, 1932.

⁴ Rapkine & Trpinac, 1939.

ACETIC FERMENTATION¹

The production of vinegar from alcoholic residues is an ancient biological industry, probably nearly as old as alcoholic fermentation itself. That it was due to a living organism was suspected as early as 1837,² though the nature of the agent was not clear till the matter was studied by Pasteur in 1862-4.³

Owing to the wide distribution of the vinegar industry many organisms have in the course of time been isolated from vinegar vats; all these are highly aerobic and all oxidise ethyl alcohol to acetic acid. This oxidation is, however, only one of many of which these organisms are capable. It was early shown by A. J. Brown, working with *Bact. aceti* and *Bact. xylinum*, that other alcohols were similarly oxidised, e.g. propyl alcohol and glycol to propionic and glycollic acids respectively, glucose to gluconic acid and mannitol to fructose.⁴ These three reactions exemplify the principal types of oxidation brought about by acetic bacteria, viz. primary alcohols and aldehydes to carboxylic acids and secondary alcohols to ketones. Strictly speaking these reactions are not fermentations but specific oxidations which stop short of completion; common usage classes them as fermentations and they are included here for convenience. Great advance in our knowledge of these oxidations was due to the classical work of Bertrand⁵ on the sorbose bacillus (since identified as *Bact. xylinum*). He isolated this organism from fermented juice of the berries of the Mountain Ash (*Sorbus aucubaria* and other varieties). After fermentation by yeast the juice usually becomes infected with the sorbose bacillus through the agency of a small red fly. This organism was found to oxidise sorbitol to the corresponding ketone sugar sorbose. Bertrand then made a detailed study of the action of the bacillus on a number of alcohols by growing it in 2% solutions of these in 5% extract of yeast. The organism was found to be highly selective, certain alcohols being attacked whilst closely related compounds were untouched; the products of oxidation were obtained in good yield, analysed and identified. Table 11 summarises the results. A comparison of the formulæ of the alcohols attacked and left respectively led Bertrand to the conclusion that only secondary alcoholic groups are vulnerable and that the group attacked must be in position 2 and adjacent to another hydroxyl group and not to a hydrogen atom:



¹ Baillie, 1916.

² Kützing, 1837.

³ Brown, 1856, 1887.

⁴ Pasteur, 1863 (2).

⁵ Bertrand, 1904.

TABLE 11

ALCOHOLS OXIDISED TO KETONES BY THE SORBOSE BACILLUS

Substrate	Product
Ethylene glycol $\begin{array}{c} \text{OH} \quad \text{OH} \\ \quad \\ \text{H}-\text{C}-\text{C}-\text{H} \\ \quad \\ \text{H} \quad \text{H} \end{array}$	Not attacked
Glycerol $\begin{array}{c} \text{H} \\ \\ \text{CH}_2\text{OH}-\text{C}-\text{CH}_2\text{OH} \\ \\ \text{OH} \end{array}$	Dihydroxyacetone $\begin{array}{c} \text{CH}_2\text{OH}-\text{C}-\text{CH}_2\text{OH} \\ \\ \text{O} \end{array}$
<i>i</i> -Erythritol $\begin{array}{c} \text{H} \quad \text{H} \\ \quad \\ \text{CH}_2\text{OH}-\text{C}-\text{C}-\text{CH}_2\text{OH} \\ \quad \\ \text{OH} \quad \text{OH} \end{array}$	<i>l</i> (+)-Erythrulose $\begin{array}{c} \text{H} \\ \\ \text{CH}_2\text{OH}-\text{C}-\text{C}-\text{CH}_2\text{OH} \\ \quad \\ \text{O} \quad \text{OH} \end{array}$
<i>i</i> -Xylitol $\begin{array}{c} \text{H} \quad \text{OH} \quad \text{H} \\ \quad \quad \\ \text{CH}_2\text{OH}-\text{C}-\text{C}-\text{C}-\text{CH}_2\text{OH} \\ \quad \quad \\ \text{OH} \quad \text{H} \quad \text{OH} \end{array}$	Not attacked
Arabitol $\begin{array}{c} \text{OH} \quad \text{OH} \quad \text{H} \\ \quad \quad \\ \text{CH}_2\text{OH}-\text{C}-\text{C}-\text{C}-\text{CH}_2\text{OH} \\ \quad \quad \\ \text{H} \quad \text{H} \quad \text{OH} \end{array}$	"Arabinulose" (araboketose) $\begin{array}{c} \text{OH} \quad \text{H} \\ \quad \\ \text{CH}_2\text{OH}-\text{C}-\text{C}-\text{C}-\text{CH}_2\text{OH} \\ \quad \quad \\ \text{O} \quad \text{H} \quad \text{OH} \end{array}$
<i>l</i> (-)-Sorbitol $\begin{array}{c} \text{H} \quad \text{H} \quad \text{OH} \quad \text{H} \\ \quad \quad \quad \\ \text{CH}_2\text{OH}-\text{C}-\text{C}-\text{C}-\text{C}-\text{CH}_2\text{OH} \\ \quad \quad \quad \\ \text{OH} \quad \text{OH} \quad \text{H} \quad \text{OH} \end{array}$	<i>l</i> (-)-Sorbose $\begin{array}{c} \text{H} \quad \text{OH} \quad \text{H} \\ \quad \quad \\ \text{CH}_2\text{OH}-\text{C}-\text{C}-\text{C}-\text{C}-\text{CH}_2\text{OH} \\ \quad \quad \quad \\ \text{O} \quad \text{OH} \quad \text{H} \quad \text{OH} \end{array}$
<i>i</i> -Dulcitol $\begin{array}{c} \text{OH} \quad \text{H} \quad \text{H} \quad \text{OH} \\ \quad \quad \quad \\ \text{CH}_2\text{OH}-\text{C}-\text{C}-\text{C}-\text{C}-\text{CH}_2\text{OH} \\ \quad \quad \quad \\ \text{H} \quad \text{OH} \quad \text{OH} \quad \text{H} \end{array}$	Not attacked
<i>d</i> (-)-Mannitol $\begin{array}{c} \text{H} \quad \text{H} \quad \text{OH} \quad \text{OH} \\ \quad \quad \quad \\ \text{CH}_2\text{OH}-\text{C}-\text{C}-\text{C}-\text{C}-\text{CH}_2\text{OH} \\ \quad \quad \quad \\ \text{OH} \quad \text{OH} \quad \text{H} \quad \text{H} \end{array}$	<i>d</i> (-)-Fructose $\begin{array}{c} \text{H} \quad \text{OH} \quad \text{OH} \\ \quad \quad \\ \text{CH}_2\text{OH}-\text{C}-\text{C}-\text{C}-\text{C}-\text{CH}_2\text{OH} \\ \quad \quad \quad \\ \text{O} \quad \text{OH} \quad \text{H} \quad \text{H} \end{array}$

As in the case of other acetic bacteria, aldehydic sugars are oxidised to acids. The oxidation of glycerol to dihydroxy-acetone has been confirmed by several observers,^{1, 2, 3} but the subject received its next impetus from Hermann and Neuschul in 1931.⁴ Thirteen well-defined strains of acetic bacteria were examined by these workers as to their oxidising powers; the results are summarised in Table 12. All the organisms oxidise ethyl and propyl

TABLE 12

	Glucose, gluconic acid	Arabinose, arabonic acid	Galactose, galactonic acid	Fructose, ketogluconic acid	Glycerol, dihydroxyacetone	Erythritol erythrulose	Mannitol, fructose	Sorbitol, sorbose	Dulcitol, galactulose	Glucuronic acid, 5-ketogluconic acid
Ketogenic										
<i>Bact. gluconicum</i> . . .	+	+	+	+	+	+	+	+	+	+
" <i>xylinum</i> . . .	+	+	+	o	+	+	+	+	o	+
" <i>xylinoides</i> . . .	+	+	+	o	+	+	+	+	o	+
" <i>orleanense</i> . . .	+	+	+	o	+	+	+	+	o	+
" <i>aceti</i> (Hansen) . . .	+	+	+	o	+	+	+	o	o	+
Aketogenic										
<i>Bact. aceti</i> (Henneberg) . . .	+	o	o	o	o	o	+	o	o	+
" <i>pasteurianum</i> . . .	+	+	+	o	o	o	o	o	o	o
" <i>acetosum</i> . . .	+	+	+	o	o	o	o	o	o	+
" <i>rancens</i> . . .	+	+	+	o	o	o	o	o	o	+
" <i>ascendens</i> . . .	+	+	+	o	o	o	o	o	o	o
" <i>vim acetati</i> . . .	+	+	+	o	o	o	o	o	o	o
" <i>kutsingianum</i> . . .	+	o	o	o	o	o	o	o	o	o
" <i>ascendens</i> . . .	+	+	+	o	o	o	o	o	o	o
" (Henneberg) . . .	o	+	+	o	o	o	o	o	o	o

* T. K. Walker, unpublished.

alcohol to acetic and propionic acids respectively; 12 oxidise glucose to gluconic acid and 11 oxidise arabinose and galactose to arabonic and galactonic acid respectively. With respect to the ketonic oxidations the group is divided fairly sharply into those capable of effecting those oxidations (the ketogenic group) and those without this power, but borderline cases exist. In addition to the ketonic oxidations shown by Bertrand others are now known to occur (see Table 13). It will be noticed that the oxidation of *d*-glucose to *d*-2-gluconic acid forms an exception to Bertrand's rule enunciated on page 103.

Not included in Table 13 is the reported production of oxalic acid by various strains of acetic bacteria from a number of compounds, chief of which are the following: glucose, fructose, galactose, maltose, sucrose, lactose, raffinose, rhamnose, arabinose,

¹ Virtanen & Bärlund, 1926.

² Bernhauer & Schön, 1928.

³ Visser't Hooft, 1925.

⁴ Hermann & Neuschul, 1931.

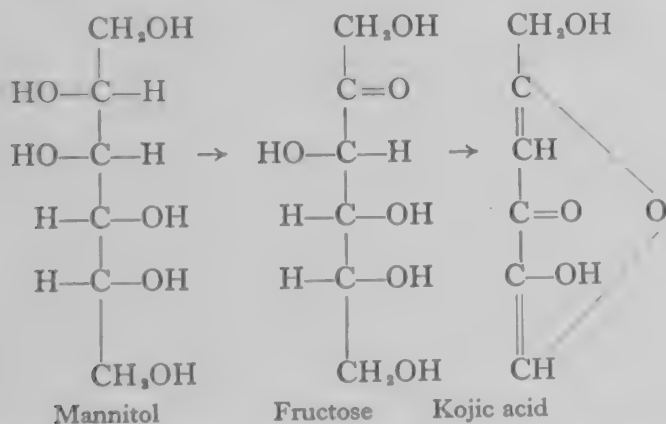
TABLE 13

Substrate	Product
Propylene glycol $\begin{array}{c} \text{H} \\ \\ \text{CH}_3-\text{C}-\text{CH}_2\text{OH} \\ \\ \text{OH} \end{array}$	Acetol ¹ $\begin{array}{c} \text{CH}_3-\text{C}-\text{CH}_2\text{OH} \\ \\ \text{O} \end{array}$
Lactic acid $\begin{array}{c} \text{H} \\ \\ \text{CH}_3-\text{C}-\text{COOH} \\ \\ \text{OH} \end{array}$	Pyruvic acid ² $\begin{array}{c} \text{CH}_3-\text{C}-\text{COOH} \\ \\ \text{O} \end{array}$
Butylene glycol $\begin{array}{c} \text{H} \quad \text{H} \\ \quad \\ \text{CH}_3-\text{C}-\text{C}-\text{CH}_3^3 \\ \quad \\ \text{OH} \quad \text{OH} \end{array}$	Acetoin ¹ $\begin{array}{c} \text{H} \\ \\ \text{CH}_3-\text{C}-\text{C}-\text{CH}_3 \\ \quad \\ \text{O} \quad \text{OH} \end{array}$
<i>i</i> -Adonitol $\begin{array}{c} \text{H} \quad \text{H} \quad \text{H} \\ \quad \quad \\ \text{CH}_2\text{OH}-\text{C}-\text{C}-\text{C}-\text{CH}_2\text{OH} \\ \quad \quad \\ \text{OH} \quad \text{OH} \quad \text{OH} \end{array}$	"Adoninulose" ³ (<i>l</i> -araboketose) $\begin{array}{c} \text{H} \quad \text{H} \\ \quad \\ \text{CH}_2\text{OH}-\text{C}-\text{C}-\text{C}-\text{CH}_2\text{OH} \\ \quad \quad \\ \text{O} \quad \text{OH} \quad \text{OH} \end{array}$
α -Glucoheptitol $\begin{array}{c} \text{H} \quad \text{H} \quad \text{OH} \quad \text{H} \quad \text{H} \\ \quad \quad \quad \quad \\ \text{CH}_2\text{OH}-\text{C}-\text{C}-\text{C}-\text{C}-\text{C}-\text{CH}_2\text{OH} \\ \quad \quad \quad \quad \\ \text{OH} \quad \text{OH} \quad \text{H} \quad \text{OH} \quad \text{OH} \end{array}$	α - <i>d</i> (-)-Glucoheptose ⁴ $\begin{array}{c} \text{H} \quad \text{OH} \quad \text{H} \quad \text{H} \\ \quad \quad \quad \\ \text{CH}_2\text{OH}-\text{C}-\text{C}-\text{C}-\text{C}-\text{C}-\text{CH}_2\text{OH} \\ \quad \quad \quad \quad \\ \text{O} \quad \text{OH} \quad \text{H} \quad \text{OH} \quad \text{OH} \end{array}$
<i>d</i> (+)-Glucose $\begin{array}{c} \text{H} \quad \text{H} \quad \text{H} \quad \text{H} \\ \quad \quad \quad \\ \text{CH}_2\text{OH}-\text{C}-\text{C}-\text{C}-\text{C}-\text{CHO} \\ \quad \quad \quad \\ \text{OH} \quad \text{OH} \quad \text{H} \quad \text{OH} \end{array}$	<i>d</i> -Gluconic acid ⁵ $\begin{array}{c} \text{H} \quad \text{H} \quad \text{H} \quad \text{H} \\ \quad \quad \quad \\ \text{CH}_2\text{OH}-\text{C}-\text{C}-\text{C}-\text{C}-\text{COOH} \\ \quad \quad \quad \\ \text{OH} \quad \text{OH} \quad \text{H} \quad \text{OH} \end{array}$
Glucose and gluconic acid	<i>d</i> (-)-5-Ketogluconic acid ⁵ $\begin{array}{c} \text{H} \quad \text{OH} \quad \text{H} \\ \quad \quad \\ \text{CH}_2\text{OH}-\text{C}-\text{C}-\text{C}-\text{C}-\text{COOH} \\ \quad \quad \quad \\ \text{O} \quad \text{OH} \quad \text{H} \quad \text{OH} \end{array}$
Glucose and gluconic acid	<i>d</i> (-)-2-Ketogluconic acid ^{5, 6} $\begin{array}{c} \text{H} \quad \text{H} \quad \text{OH} \\ \quad \quad \\ \text{CH}_2\text{OH}-\text{C}-\text{C}-\text{C}-\text{C}-\text{COOH} \\ \quad \quad \quad \\ \text{OH} \quad \text{OH} \quad \text{H} \quad \text{O} \end{array}$
Glucose	6-Aldehydogluconic acid ^{7, 8} (guluronic acid) $\begin{array}{c} \text{H} \quad \text{H} \quad \text{OH} \quad \text{H} \\ \quad \quad \quad \\ \text{CHO}-\text{C}-\text{C}-\text{C}-\text{C}-\text{COOH} \\ \quad \quad \quad \\ \text{OH} \quad \text{OH} \quad \text{H} \quad \text{OH} \end{array}$

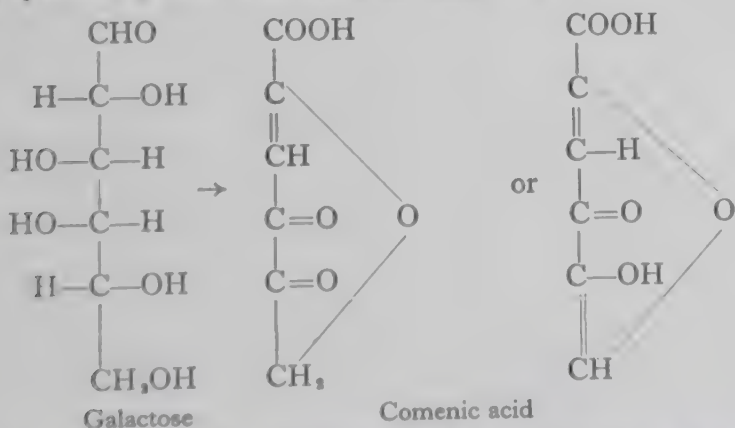
¹ Kling, 1901.³ Visser't Hooft, 1925.⁵ Bernhauer & Gorlich, 1935 (1).⁷ Takahashi & Asai, 1931.² Cozic, 1933.⁴ Bertrand & Nitzberg, 1928 (1), (2).⁶ Bernhauer & Knoblauch, 1939⁸ Bernhauer & Irrgang, 1935.

ethyl alcohol, ethylene glycol, acetic, glycolic, lactic and malonic acids. The chemical evidence rests mainly on the crystalline form of the calcium salt isolated and is not conclusive, but the work cannot be disregarded without further investigation.¹

A reaction peculiar to certain species of acetic bacteria isolated in Japan² produces kojic acid in small yield from fructose and mannitol (0.105 g.-0.755 g. from 10 g. fructose). The only other species known to carry out this reaction is *Bact. xylinoides*, and then in extremely small yields. The course of the reaction is unknown but is certainly different from that of the *Aspergillaceæ* where it also occurs, since the bacteria cannot utilise 2- or 3-carbon compounds for this synthesis.



The formation of comenic acid from galactose has been reported for a Japanese species *Gluconobacter nov. sp.*³



As has already been mentioned, the production of acetic acid from alcohol is a strictly aerobic process. Neuberg and Windisch⁴ have shown that three separate acetic bacteria (*B. ascendens*, *B.*

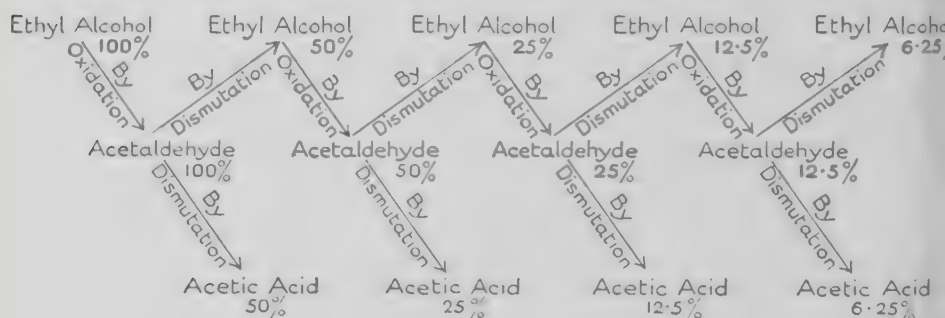
¹ Barming, 1902.

³ Takahashi & Asai, 1936.

² Takahashi & Asai, 1933.

⁴ Neuberg & Windisch, 1925.

pasteurianum and *B. xylinum*) may act anaerobically on acetaldehyde with the production of equimolecular proportions of ethyl alcohol and acetic acid. Heavy suspensions of bacteria equivalent to 0.7 g. dry weight in a litre of saline were allowed to act on 0.2% of acetaldehyde in the presence of calcium carbonate, and the whole mixture saturated with carbon dioxide; that is, the reaction proceeded in a saturated solution of calcium bicarbonate. The conditions were anaerobic, and the view that molecular oxygen was playing no part was supported by the fact that the process was unaffected by cyanide. Aerobically 50% only of the acetaldehyde is dismuted in this way, whilst 50% is oxidised direct to acetic acid; thus in these conditions acetaldehyde gives rise to 75% of acetic acid and 25% ethyl alcohol. Neuberg and Windisch have suggested that where the oxidation proceeds in well-aerated conditions the process may be one of alternate oxidation and dismutation until the whole of the alcohol becomes oxidised to acetic acid. This is represented diagrammatically below:



It has been shown, however, by Wieland and Bertho,¹ that though a Cannizzaro reaction does actually take place in the conditions described, this reaction is probably of little importance in the production of acetic acid from alcohol by the living cell, owing to the extreme slowness with which it proceeds in comparison with direct oxidation. They showed that in the presence of suspensions of *B. orleanense* both ethyl alcohol and acetaldehyde could be oxidised by molecular oxygen to acetic acid; also, repeating the work of Neuberg and Windisch, that with the same organism in strictly anaerobic conditions a dismutation took place. In the latter experiment 1 ml. of bacterial suspension (15.8 mg. dry weight) with 1 ml. *M*/5 buffer at pH 5.6 + 6 ml. water + 1 ml. 0.4 *M* acetaldehyde was dismuted to ethyl alcohol and acetic acid. A similar experiment was then performed aerobically and the oxygen uptake measured. In 160 minutes 80% of the

¹ Wieland & Bertho, 1928.

aldehyde was oxidised, the reaction being complete in 340 minutes, 24.6 mg. of acetic acid being formed as against an expectation from theory of 24.0 mg. with an oxygen uptake of 4.48 ml. A direct comparison of the rates of the two processes of dismutation and oxidation showed that in the former 0.138 mg. of alcohol was formed in 2.5 hours. This would require 33.6 μ l. of oxygen for oxidation; hence if the oxidation of acetaldehyde were proceeding by way of a dismutation process the rate of oxygen uptake should not exceed this figure. Actually, however, in aerobic experiments the uptake in the same time amounted to 1300 μ l., i.e. forty times as much. Results of a similar order were obtained with *Bact. pasteurianum*. According to Simon,¹ however, the relative rates of dismutation and oxidation depend somewhat on reaction. The earlier work on which the dismutation theory was based was done at pH 8.1 (in the presence of calcium carbonate), whilst that of Wieland and Bertho was done at pH 5.5. At the more acid reaction dismutation was found to be at a minimum, whilst at the alkaline reaction alcohol was produced from acetaldehyde in amount equal to 8–10% of theory.

This has been further confirmed by a study of the alcohol and acetaldehyde dehydrogenases and the aldehyde mutase of *Bact. ascendens*.²

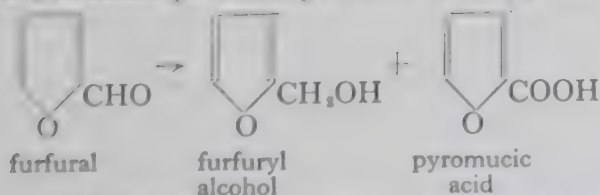
Table 14 shows clearly that at acid reactions dismutation is at a minimum. The slight variation in pH optima of the two

TABLE 14³

	Young cults. pH optimum	Old cults. pH optimum
Alcohol dehydrogenase . .	5.57–6.0	5.93–5.7
Aldehyde dehydrogenase . .	5.9–6.5	6.3–6.6

Aldehyde mutase optimum above pH 8.4, upper limit not determined on account of polymerisation of acetaldehyde.

dehydrogenases has as a result the accumulation of acetaldehyde in aerobic conditions with young cultures at pH values lower than 6.0. Dismutations similar to that occurring with acetaldehyde occur with other aldehydes, viz. *n*-butaldehyde, *iso*-valeraldehyde, propaldehyde, furfural (to furfuryl alcohol and pyromucic acid):⁴



¹ *Enzymes*, 1932. ² Janke & Kropatsch, 1935. ³ *Ibid.*, ⁴ Molinari, 1929.

CHAPTER V

NITROGEN METABOLISM

Nitrogenous excretory products

Many bacterial cells excrete into the medium nitrogenous products of high molecular weight, both proteins and polypeptides. Some of the former are known to be enzymes. The earlier ones to be recognised as such were those which had their counterparts in animals and plants, such as amylase, maltase, invertase and various proteases. Others were first recognised in the bacterial filtrates as possessing poisonous properties and were known as toxins. Some of these have now been recognised as enzymes attacking vital tissues of their hosts, and thus exerting their toxic action. Other toxic proteins may in time be shown to be enzymes, but there may be others which owe their toxicity to other forms of action. In addition there are toxic polypeptides, the mechanism of whose action remains to be discovered.

Proteolytic enzymes

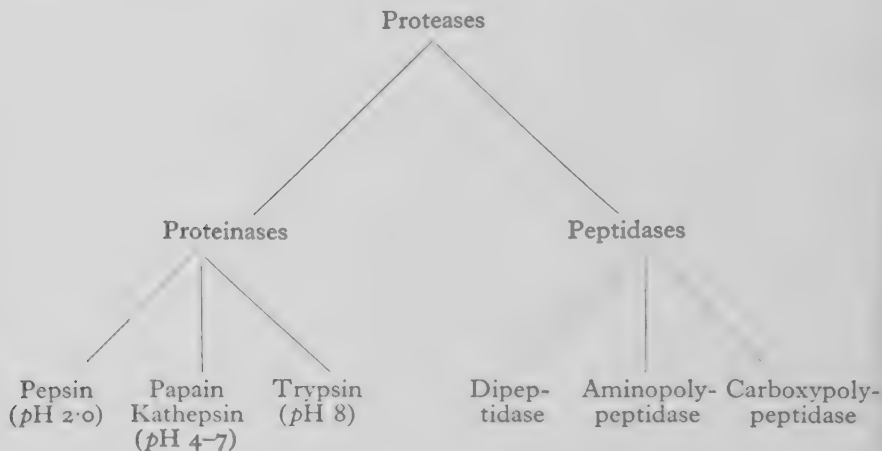
The ability to break down protein to peptones, polypeptides and amino-acids is not shared equally by all groups of bacteria. It is in the main achieved by exoenzymes excreted into the medium—a necessary result of the impermeability of the cell to the protein molecule. The families in which proteolytic enzymes most frequently occur are the *Pseudomonadaceæ*, the *Bacillaceæ*, both aerobic and anaerobic, and some tribes of the *Enterobacteriaceæ*, in particular the *Proteæ*, and the *Clostridia*. In putrefactive species, depending for their food supply on the decomposition of proteins, it is obviously necessary that some extracellular mechanism be present to disrupt the protein molecule into diffusible constituents. Early work¹ showed that a number of species liquefied gelatin and fibrin, a property still possessed by the culture media after the removal of the cells. The liquefaction of gelatin has long been the routine diagnostic test for proteolysis. The presence of fermentable carbohydrate in the culture media inhibits the production of proteases, but whether this action is due to the production of acid or to the inhibition of the formation of proteolytic enzymes is not certain.

Proteases were first studied in the digestive juices of animals and

¹ Brunton & Macfadyen, 1889.

in preparations from plants; only recently have studies been made on those of bacterial origin. The early attempt to classify proteases rested primarily on the size of the molecule attacked, whole proteins being attacked by "proteinases" and peptides by "peptidases." The former group were differentiated mainly by the optimum pH of their activity, the latter by the character of the free group, $-NH_2$ or $-COOH$, of the substrate attacked.

The following scheme due to Grassmann and Schneider¹ illustrates this:



More recent work, largely due to Bergmann,² shows that proteases are not satisfactorily classified on the basis of the size of the molecule they attack. Peptidases, besides attacking di- and polypeptides, can attack the peptide links of the protein molecule provided that these conform to certain structural requirements. Thus aminopeptidases attack only those links adjacent to a free amino group; they require also that the amino-acid side chain on the carbonyl side of the peptide link shall be leucyl. Provided that these conditions are fulfilled the link can be ruptured whether it occurs at the end of a protein or of a peptide molecule. Carboxypolypeptidases attack only those links adjacent to a free carboxyl, and the amino-acid side chain on the imino side of the peptide link must be tyrosyl or phenylalanyl. Linkages fulfilling these requirements are ruptured both in protein molecules and in peptides. Enzymes which used to be classified as proteinases can rupture not only the internal and terminal links of protein molecules, but also those of peptide molecules, but their activity is also governed by the nature of the amino-acid side chains adjacent to the links which are ruptured. Thus trypsin requires the basic arginyl and lysyl groups attached to the carbonyl side of the peptide link attacked;

¹ Grassmann & Schneider, 1936.

² Bergmann, 1942.

pepsin requires the aromatic tyrosyl or phenylalanyl residues attached to the imino side of the link.

Work along these lines has not so far been done for bacterial proteases so a satisfactory classification of these enzymes is not yet possible.

Fig. 1 illustrates the points of attack of the various enzymes on the protein molecule.

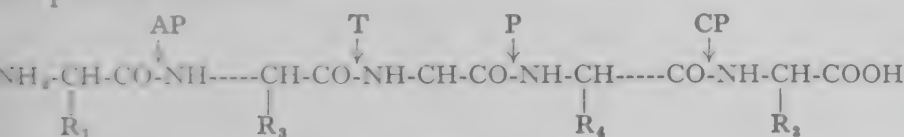


FIG. 1

R_1 = leucyl; R_2 = tyrosyl or phenylalanyl; R_3 = arginyl or lysyl; R_4 = tyrosyl or phenylalanyl; AP = aminopolypeptidase; CP = carboxypolypeptidase; P = pepsin; T = trypsin.

A further basis for the differentiation of proteases lies in their activation by metals Fe, Mn and Mg and by reducing agents such as ---CN or ---SH .

Quite roughly speaking proteolytic bacteria appear to liberate enzymes acting on large protein molecules early in the growth period. Peptidases acting only on smaller molecules tend to appear later, probably as a result of autolysis. Thus in the group of cheese-ripening organisms known as acidoproteolyten (on account of the fact that they produce both lactic acid and a filterable protease) a filterable proteinase was obtained in the culture fluid and three types of peptidase from the dried bacterial cells.¹ From the culture medium of *B. pyocyaneus* a filtrate was obtained attacking gelatin, silk peptone, leucylglycine and leucylglycylglycine; the enzyme complex attacking the peptides was dialysable, adsorbed on kaolin and alumina and eluted by acetic acid.²

A series of studies on the proteases of bacterial filtrates is due to Maschmann.³ The proteases produced by the organisms which Maschmann studied fall into at least four groups, as shown in Table 1. The oxygen-stable enzymes are extracellular and the oxygen-labile enzymes, which are activated by reducing compounds, are probably intracellular since they appear in the culture fluid only after growth has reached its maximum.

The specific gelatinases are of particular interest since they apparently attack only gelatin and its precursor collagen. Maschmann suggested that the invasiveness of the gas-gangrene organisms may in part be due to this enzyme. More recently the effect of the collagenase of *Cl. welchii* has been studied in wounds and on isolated muscle tissue.⁴ Muscle treated with culture filtrates

¹ Gonn, Grassmann & Schleich, 1932.

² Gorbach, 1930.

³ Maschmann, 1937 (1), (2), (3); 1938 (1), (2), (3).

⁴ Robb-Smith, 1945.

TABLE 1

Organism*	Proteases			
	Oxygen-stable		Oxygen-labile	
	Specific gelatinases	Proteinases inhibited by normal serum	Proteinases inhibited by normal serum	Peptidases
<i>B. prodigiosus</i> . .	—	+	—	+
<i>B. fluorescens</i> . .	—	+	—	+
<i>B. pyocyaneus</i> . .	—	+	—	+
<i>B. botulinus</i> . .	—	+	+	+
<i>B. chauveoi</i> . .	+	—	—	+
<i>B. histolyticus</i> . .	+	+	+	+
<i>B. welchii</i> . .	+	—	+	+
<i>Vibrio septicus</i> . .	+	—	+	+
<i>B. sporogenes</i> . .	—	+	+	+
<i>B. tetani</i> . .	—	+

* Maschmann's nomenclature

of *Cl. welchii* gave a friable mass of fibrils from which the supporting tissue and sarcolemma had disappeared. The same tissue treated with trypsin gave a gelatinous mass with the fibrils dissolved and the connective tissue intact. That the former action is specifically due to the collagenase and not to the α -toxins or the hyaluronidase of *Cl. welchii* has been shown by immunological means.¹

If the invasiveness of *Cl. welchii*, *Cl. histolyticum*, *Cl. septicum* and *Cl. chauveoi* is in part due to the specific gelatinases then the fact that these enzymes are not inhibited by the trypsin inhibitor, which is present in normal serum, may be significant. The main proteinase of the non-invasive pathogens *Cl. botulinum*, *Cl. tetani* and *Cl. sporogenes* is inhibited by normal serum. This group of oxygen-stable extracellular proteinases is able to digest a wide variety of proteins, including gelatin, as are the serum-inhibited oxygen-labile (i.e. —SH activated) proteinases which are apparently intracellular. The intracellular peptidases (aminopeptidases and dipeptidases) require metals (Fe, Mn) as well as reducing compounds for their activation.

The character of the proteinases of the *Clostridia* may show considerable variation due to strain; thus Maschmann² reported from the filtrate of *Cl. histolyticum* (*B. histolyticus*) a proteinase slightly inhibited by cysteine whilst Kocholaty *et al.*³ and van Heyningen⁴ separately reported culture filtrates that were activated by cysteine. The latter worker, however, employed a strain which on ageing threw a variant with an extracellular enzyme in-

¹ Oakley *et al.*, 1946.³ Kocholaty *et al.*, 1938 (1).² Maschmann, 1938 (1).⁴ van Heyningen, 1940.

hibited by cysteine (agreeing with Maschmann) and produced a second enzyme—probably due to autolysis—which was activated by cysteine. This is but one example of the apparently erratic and variable nature of the production of exoenzymes by the *Clostridia*.

A proteolytic enzyme is produced by some group A streptococci which attacks the M antigens of all group A organisms tested. This antigen is an acid-soluble nucleoprotein. As a rule cultures producing the enzyme lack the M antigen when grown at 37°, though it is sometimes present when the cells are grown at 22°. The enzyme has been concentrated by precipitation by ammonium sulphate; it attacks fibrin, streptococcal fibrinolysin, casein, gelatin and benzoyl-*l*-arginine-amide, and in general resembles papain and some cathepsins. It is active only in the presence of reducing agents.¹

The effect of media on the production of filterable proteases

Earlier investigators reported the frequent absence of filterable proteases when organisms were cultivated in synthetic media, but the literature contains many contradictions.^{2, 3, 4, 5} The fact that filterable proteases can be produced on simple synthetic media has finally been established by several workers.^{6, 7, 8} Highly thermostable proteases have been reported from *B. fluorescens liquefaciens*.⁹

Merrill and Mansfield Clark,¹⁰ working with a strain of *Proteus*, reported that on a synthetic medium, calcium and magnesium were necessary for the production of the proteolytic enzyme; this was confirmed by Haines but not by Wilson (working with *Ps. pyocyanea*). Haines, moreover, demonstrated that the effect of magnesium was on growth but not on gelatinase production, but that calcium had little effect on growth but actively stimulated gelatinase production. These observations are probably linked with the activation of proteases by metals.

Evidence that there are many unknown factors concerned with the production of proteolytic enzymes is supplied by recent work on *Bac. subtilis*. On a medium fortified with bran, yeast and malt extract 10,000 gelatinase units per ml. were produced compared with 20–100 units per ml. on horse-meat broth.¹¹

Intracellular proteases

Proteolytic enzymes exist also inside the cell and are more widely distributed than the exoenzymes. In general the endo-

¹ Elliott, 1945. ² Fermi, 1890. ³ Jordan, 1906. ⁴ Drummond, 1914.
⁵ Diehl, 1919. ⁶ Wilson, 1930. ⁷ Merrill & Mansfield Clark, 1928.
⁸ Haines, 1931, 1932, 1933. ⁹ Virtanen & Tarnanen, 1932 (1).
¹⁰ Merrill & Mansfield Clark, 1928. ¹¹ Ramon *et al.*, 1945.

enzymes act on the smaller molecules. Various methods have been used for disrupting the cell, such as 40% glycerol,¹ bile at 0° acting on the pneumococcus,² powdered glass on *Str. hæmolyticus*.³ Otsuka⁴ compared the proteolytic power of *Staph. aureus* and *B. prodigiosus* killed by toluene with the Chamberland filtrates of the same organisms, using as substrates gelatin and serum on the one hand and glycyl-*l*-tyrosine and glycyl-*l*-tryptophan on the other. Both the filtrate and the cells attacked the proteins but only the cells attacked the dipeptides. *Bact. coli* similarly treated was inactive to both proteins and dipeptides. In this connection the early work of Taylor⁵ may be cited. Here casein was inoculated with *Bact. coli* and incubated for five months; by this time 76% of the combined phosphorus had been split off but no amino-acids formed. This organism therefore seems to be unable to attack proteins so as to produce any cleavage products sufficiently degraded to be available to it; such organisms if grown on proteins require them to be previously digested to the amino-acid stage.⁶ This view is confirmed by the work of Rettger *et al.*,⁷ in the course of which they grew non-proteolytic members of the *coli* group on Witte's peptone, and followed the disappearance of the proteoses and peptones by the biuret test adapted to give roughly quantitative results. No marked disappearance of biuret products could be shown, though with proteolytic bacteria these disappeared in 1-2 weeks. It must, however, be remembered that these cells were not disintegrated so the experiment affords no information on the intracellular enzymes.

The action of bacteria on pure proteins and on their degradation products

Of great importance relative to the previous discussion is the immunity of pure proteins from bacteriological attack, even by highly proteolytic species. The first observation on this subject was made by Bainbridge,⁸ who used crystallised egg albumin, serum proteins and alkali albumin in concentration 0.1-0.5%, together with the usual salts, but no other source of nitrogen or carbon. Into this medium he sowed the following organisms: *B. coli communis*, *B. enteritidis*, *B. typhosus*, *B. proteus*, *B. pyocyaneus*, *Staph. aureus* and gonococcus. In spite of the fact that highly proteolytic organisms were included in these experiments, no growth (as tested by bacterial counts) and no degradation of proteins were obtained; if impure gelatin was used slight growth took place with *B. pyocyaneus*, *Staph. aureus* and *B. proteus* (the most

¹ Macfadyen, 1892. ² Avery & Cullen, 1920. ³ Stevens & West, 1922.

⁴ Otsuka, 1916. ⁵ Taylor, 1902. ⁶ See also Berman & Rettger, 1918.

⁷ Rettger *et al.*, 1916. ⁸ Bainbridge, 1911.

proteolytic of the collection), the growth of *B. pyocyaneus* and *B. proteus* being further improved by the addition of glucose. When peptone was added *B. proteus* readily attacked the proteins. These observations were confirmed and enlarged by Sperry and Rettger.¹ The organisms used included some of the most proteolytic known, both aerobes and facultative and strict anaerobes, and also species known to grow easily on synthetic media; the proteins used were crystallised serum and egg albumins and crystallised edestin; the salts were those usually employed, excluding ammonium. No growth and no decomposition of the proteins occurred. If, however, a small amount of peptone was added to the medium multiplication started and the proteins were rapidly broken down. The authors of these observations advanced the view that bacteria require peptone in order to synthesise their proteolytic enzymes and that in its absence they starve, as it were, for lack of the key with which to unlock the larder. This explanation is, however, not proved. In the first place, it is now certain that all proteolytic bacteria do not require peptone or some form of organic nitrogen in order to synthesise diffusible proteolytic enzymes (see p. 115). Moreover the observed phenomena seem perfectly explicable on the assumption that the primary cleavage of the protein molecule is the work of a diffusible enzyme. When cells are sown into fresh media this enzyme becomes very highly diluted, and its action may easily be so retarded as to be practically negligible. In the absence of any means of concentrating the diffusible enzyme, the cells are deprived of the only means of initiating their attack, and the pure protein remains unaltered. The part played by the peptone may therefore be that of an available food supply rather than a special enzyme producer, and on this view might conceivably be replaced by any adequate synthetic medium, provided the reaction remained within the limits at which the proteolytic enzymes could function.

Not only are pure native proteins, when serving as the sole food supply, immune from bacteriological attack, but the same is true of coagulated proteins² and proteoses;^{3, 4} the last-named material was prepared from commercial peptone by precipitation with ammonium sulphate and purified by dialysis. In the presence of an additional source of food (Liebig's meat extract 0.5%) the proteose was attacked by *B. subtilis*, *B. ramosus*, *B. proteus* and *B. prodigiosus*, as shown by the gradual disappearance of substances giving the biuret reaction; it was not, however, attacked by *Staph. aureus* and *albus* nor by members of the *Bacteriaceae*.

It is clear from the above data that the liquefaction of gelatin

¹ Sperry & Rettger, 1915.

² Rettger, Berman & Sturges, 1916.

³ Berman & Rettger, 1916, 1918.

⁴ Ibid.

does not always connote far-reaching proteolytic activities. This is, for example, the case with *Staph. aureus* and *Bact. cloacæ*, which both liquefy gelatin but are not able to decompose it to abiuret substances nor to attack proteoses nor coagulated proteins. It seems in the case of these organisms that the possession of the exoenzyme is no criterion of far-reaching proteolytic activity, nor, so far as one can see, of any service to the cell.

Casein, like gelatin and other proteins, is attacked by the other proteolytic organisms already mentioned, but only in the presence of an extraneous food supply (Liebig's meat extract was used); *Staph. aureus* and *albus* and the members of the *Bacteriaceæ* group were without action upon it.

These facts show that for non-proteolytic bacteria the larger units in commercial peptone are useless, these organisms being able to use only amino-acids and possibly the smaller polypeptides; the extensive use of protein digests as laboratory media is thus justified.

The toxic proteins

Many proteins excreted by bacteria are intensely toxic and in some cases their mode of action is understood. One of the earliest of these to be studied was hyaluronidase, first known as the "spreading factor." This is an enzyme attacking hyaluronic acid and is dealt with under polysaccharide decomposition in Chapter III.

The next toxin whose action has been explained is the α -toxin of *Cl. welchii*. Nagler¹ first observed that toxic filtrates from this organism produced on serum and on egg yolk an opalescence followed by the separation of a cream; the degree of opalescence on egg yolk as measured nephelometrically being used, in association with the determination of the M.L.D. on the mouse, as a convenient method of assaying the toxin.² The toxin itself was partially purified by adsorbing on to calcium phosphate in the presence of acetone and eluting with ammonium sulphate. Acting on lecithin it hydrolyses the linkage at *A*, leaving phosphoryl choline and stearyl oleyl diglyceride (Fig. 2).³ Snake venoms, it is interesting to note, attack lecithin at *B*, splitting off oleic acid and leaving a hæmolytic compound, lysolecithin. Another lecithinase present in rice bran attacks the lecithin at *B* and *C*; lysolecithin is also attacked at *C*, the loss of the stearyl radicle annulling its hæmolytic properties. It has been claimed that *Vibrio comma* attacks lecithin at *A*, *B*, *C* and *D*, producing free stearic and oleic acids, choline and phosphoric acids.⁴

¹ Nagler, 1939.

³ Macfarlane & Knight, 1941.

² van Heyningen, 1941.

⁴ Felsenfeld, 1944.

The α -toxin—probably in virtue of its property of disrupting lecithin—is a hæmolysin, but many bacterial hæmolysins exist whose method of disrupting red blood cells is unknown.

Two important and extremely potent toxins have recently been isolated in a state of purity and crystallised, but so far their mode of action is unexplained.

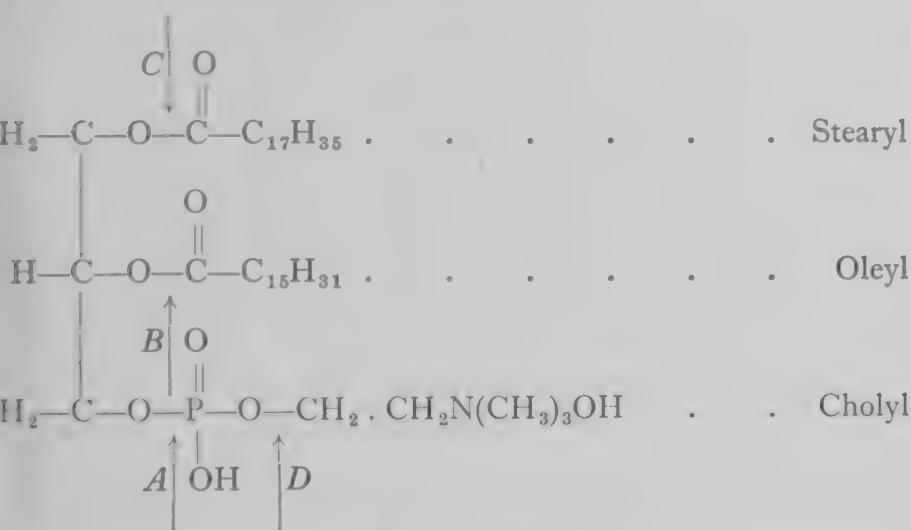


FIG. 2.—Lecithin

The toxin from *Cl. botulinum* (type A) was purified from a medium containing casein 3%, glucose 0.5% and 1% corn-steep liquor containing about 50% solids and treated with alkali. After growth of the organism 16 l. medium contained 800 mg. toxin, of which 40–160 mg. was isolated. The product was finally crystallised from sodium acetate and sodium chloride at pH 4 in needle-shaped plates. The toxicity of this product is excessively high, LD₅₀ being 0.000032 μ g. when injected intraperitoneally. It was computed that, for a man of 75 kilos having the same susceptibility as a mouse, the LD₅₀ would be 0.12 μ g. Thus a food containing 1×10^6 MLD./ml. would be fatal in a dose of 0.004 ml.¹

The tetanus toxin was obtained on a semisynthetic medium. The toxin present in the crude filtrate was 6 mg./l. It was obtained by precipitation with methanol in controlled conditions in 27–60% yield. The product is a crystalline protein, electrophoretically homogeneous, containing 50–75 million MLD./mg.N.²

The diphtheria toxin has been prepared by the action of *C. diphtheriae* on a chemically defined medium containing no protein. From the culture filtrate a protein was isolated which was charac-

¹ Lamanna *et al.*, 1946.

² Pillemer *et al.*, 1946.

terised and appeared to be identical with the toxin and had a toxicity of 10^7 /M.L.D./g.¹

The production of toxin is closely dependent on the concentration of iron in the medium; at very low iron concentrations both growth and toxin production are poor; as iron is added both increase, toxin production attaining a maximum at 0.1 mg. of iron per litre.² As more iron is added toxin falls off steeply though growth increases slightly; at a level of 0.6 mg. iron per litre no toxin appears (see p. 179). Along with the toxin, porphyrin is found in the filtrate and the production of both is inhibited by the presence of iron above 0.1 mg./litre, 4 atoms of iron preventing the formation of 4 molecules of porphyrin and 1 molecule of toxin; simultaneously the intracellular content of iron rises.

The interpretation put on these striking phenomena is that in the presence of adequate supplies of iron the growing cell synthesises an iron-porphyrin respiratory intracellular pigment; when the iron supply fails the cell continues to synthesise the porphyrin and specific protein portions of the molecule which are excreted into the medium, the specific protein being the diphtheria toxin.³

Non-enzymic nitrogenous excretions : gramicidin and tyrocidin

Two polypeptides of interest are excreted by the aerobic spore bearer, *Bac. brevis*, and there are indications that there are others. The organism was isolated by Dubos from soil heavily and repeatedly inoculated with living gram-positive bacteria; this was done with the object of using a selective medium to obtain an organism with a mechanism for lysing or destroying gram-positive bacteria.⁴ From the culture filtrate of *Bac. brevis* an active material, tyrothricin, was obtained appearing in combination with proteins, from which it is liberated by precipitation of the protein by acid acetone or by proteolytic enzymes.⁵ Tyrothricin contains two polypeptides, gramicidin and tyrocidin, both antibacterial in action and both insoluble in water and soluble in certain organic solvents.⁶ Gramicidin can be crystallised from acetone and is soluble also in ethanol, glacial acetic acid and dioxan. It has no free $-\text{NH}_2$ or $-\text{COOH}$ groups⁷ and contains 45%⁸ of its amino-acids in the *d*- or "unnatural" configuration. It is a cyclopeptide with 24 residues. Acid hydrolysis⁹ and chromatographic analysis¹⁰ show that these are: 6 *d*-leucine, 6 *l*-tryptophan, 5 *d*- and *l*-valine, 3 *l*-alanine, 2 glycine and 2 ethanolamine. The second polypeptide, tyrocidin, can be crystallised from methanol or

¹ Pappenheimer *et al.*, 1937.

³ Pappenheimer, 1947.

⁶ Hotchkiss & Dubos, 1941.

⁸ Lipmann *et al.*, 1941.

² Pappenheimer & Johnson, 1936, 1937.

⁴ Dubos, 1939 (1).

⁵ *Ibid.* (2).

⁷ Hotchkiss, 1941.

⁹ Gordon *et al.*, 1943 (1).

¹⁰ Synge, 1945 (1), (2).

ethanol, is strongly basic and is active against both gram-positive and gram-negative bacteria. The following amino-acids have been identified in its acid hydrolysate: *d*-phenylalanine, *l*-leucine, *l*-valine, *l*-proline, *l*-tyrosine, *l*-glutamic acid, *l*-ornithine, *l*-aspartic acid and *l*-tryptophan.¹

Apart from the antibiotic properties of these polypeptides² the excretion by the cell of well-defined compounds containing *d*-amino-acid residues raises problems of great biological interest. It is possible that both polypeptides³ may owe their toxicity in some measure to these units. Tyrocidin appears to act also as a cationic detergent. Its effect on *Staph. aureus* and *Str. hæmolyticus* is to release N and P compounds from the cell. A similar and more striking effect is reported for *Str. fæcalis* where the effect of tyrocidin is to cause the sudden and complete passage of lysine and glutamic acid from the internal to the external environment of the cell. Similar effects were found with the detergents cetyl trimethyl ammonium bromide and aerosol O.T. but not with gramicidin.⁴ Examination of cells thus treated with the electron microscope disclosed rupture and curling of the cell membrane.⁵

Closely related to tyrocidin is gramicidin S (Soviet gramicidin) excreted by a soil bacillus and reported by Sergieff.⁶ This is a cyclic polypeptide having the following sequence occurring once or in an endless peptide chain (cyclopeptide):^{7, 8}

-*z*-*l*-valyl-*l*-ornithyl-*l*-leucyl-*d*-phenylalanyl-*l*-prolyl-*d*-(-)glutamic acid occurs as the sole amino-acid of the protein capsule of *Bac. anthracis*⁹ and of a polypeptide excreted by *Bac. subtilis*.¹⁰

AMINO-ACID BREAKDOWN

Amino-acids of the general formula $R \cdot CHNH_2 \cdot COOH$ are deaminated by all groups of bacteria except the majority of the staphylococci and streptococci; the ammonia thus liberated forms the principal source of nitrogen for bacterial growth. Amino-acids may also be decarboxylated with the production of amines, the two processes forming alternative methods of breakdown, deamination occurring in cells grown at a neutral or alkaline pH and decarboxylation as a response to an acid environment.

Deamination

Bacterial deamination proceeds by several methods which differ according to the enzyme make-up of the organism in question and to the conditions prevailing in the medium.

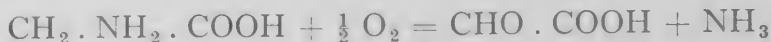
¹ Gordon *et al.*, 1943 (2). ² Hotchkiss, 1944. ³ *Ibid.*, in preparation.

⁴ Gade & Taylor, 1946. ⁵ *Ibid.* ⁶ Sergieff, 1943, 1944. ⁷ Sygne, 1945.

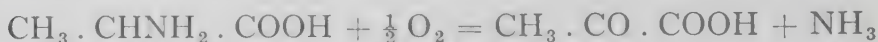
⁸ Combs *et al.*, 1946. ⁹ Hanby & Rydon, 1946. ¹⁰ Bovernick, 1942.

1. *Oxidative deamination*

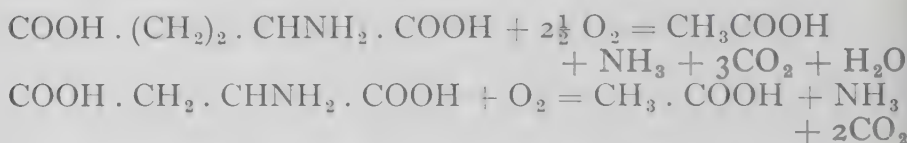
This results in the formation of a keto-acid. Using intact cells this reaction can only be demonstrated in the presence of an inhibitor such as toluene or nitrite to prevent the further decomposition of the keto-acid. With purified enzymes extracted from the cell a fixative for the keto-acid is required, which otherwise exerts a toxic action on the enzyme and quickly brings the reaction to a standstill. The oxidative deamination of glycine in the presence of 2,4-dinitrophenylhydrazine has been shown in the case of *Ps. fluorescens*, *Bac. mycoides*, *Bact. coli* and *Bact. vulgare*;¹ glyoxylic acid was identified, the reaction conforming to the equation:



A similar study on *dl*-alanine with a soil organism resulted in the accumulation of pyruvic acid.² The same reaction carried out with *Esch. coli* treated with toluene proceeded quantitatively according to the equation:³



The oxidative breakdown of *l*-(−)aspartic acid and *l*-(+)glutamic acid by *H. parainfluenzæ* results in the production of acetic acid and CO₂.



This organism requires coenzyme I or II as a growth factor. If grown in suboptimal amounts of coenzyme the resulting cells are unable to carry out the oxidations at the optimal rate unless coenzyme is added to the system. The products obtained are obviously the result of a series of reactions following the initial oxidation.⁴

A cell-free enzyme (or group of enzymes) has been obtained from *Proteus vulgaris* after the disintegration of the cells by supersonic waves, and subsequent removal of cell debris. The *l*-amino-acids attacked were *nor*-leucine, phenylalanine, leucine, tryptophan, methionine, tyrosine, *nor*-valine, histidine, arginine, *isoleucine* and α -amino-butyric acid. The keto-acids were identified in each case, though whether the reactions were all catalysed by the same enzyme is not clear.⁵

¹ Janke & Tayenthal, 1936.² Aubel & Egami, 1935 (1).³ Unpublished.⁴ Klein, 1940.⁵ Stumpf & Green, 1944.

2. Deamination and desaturation

The reaction $R \cdot CH_2 \cdot CHNH_2 \cdot COOH \rightarrow R \cdot CH:CH \cdot COOH + NH_3$ was first observed in the case of the change of *l*-histidine to urocanic acid¹ through the prolonged action of a number of organisms on *l*-histidine in Ringer's solution. A similar observation was made on the desaturation of *l*-tyrosine by *B. proteus*.² The only other amino-acid so far shown to undergo this type of deamination is aspartic acid. Under the influence of washed

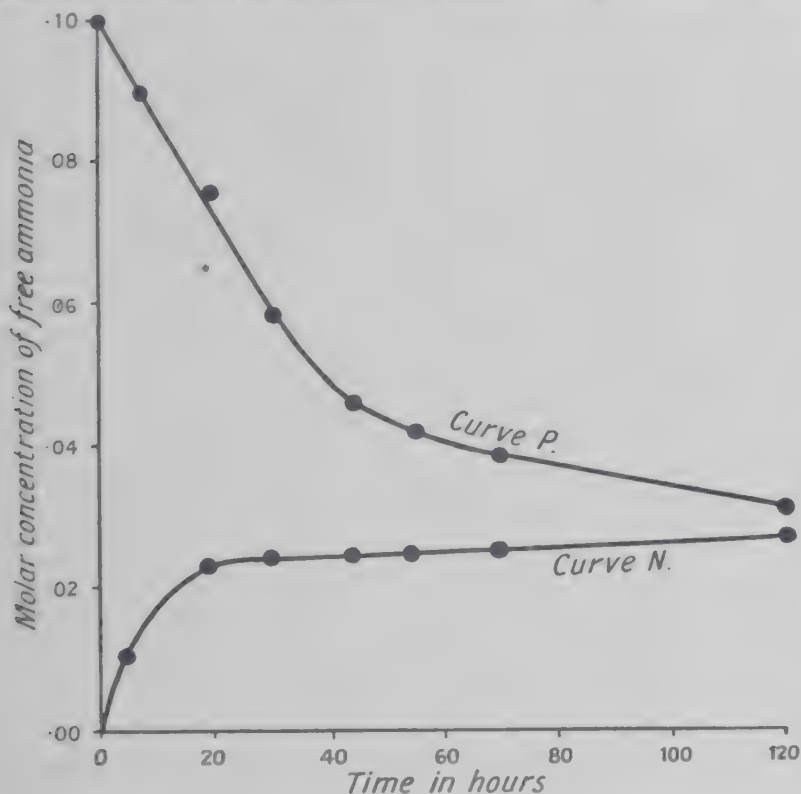


FIG. 3

suspensions of *Bact. coli* and some other facultative anaerobes the reversible reaction $\text{aspartic acid} \rightleftharpoons \text{fumaric acid} + NH_3$ has been demonstrated^{3, 4} (see Fig. 3). In order to achieve this, fumaric acid and ammonia on the one hand and aspartic acid on the other are incubated together in the presence of washed suspensions of the organism previously treated with 2% cyclohexanol. The anti-septic inhibits the enzyme fumarase also present in the cell, catalysing the reaction



and enables the true fumaric-aspartic equilibrium to be reached.⁵

¹ Pasteur, 1917.

² Hira, 1921.

³ Cook & Woolf, 1928.

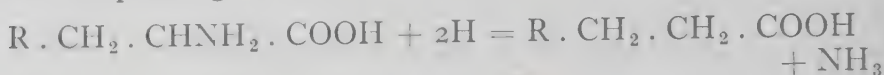
⁴ Woolf, 1929.

⁵ Ibid.

A second enzyme (aspartase II) obtained in a cell-free condition from *Bact. coli* differs from the first in being inhibited by toluene and in requiring a coenzyme which can be replaced by (and may be identical with) adenosine.¹

3. Reductive deamination

In general the compound resulting from deamination by a strict anaerobe or by a facultative anaerobe acting anaerobically is the corresponding saturated fatty acid



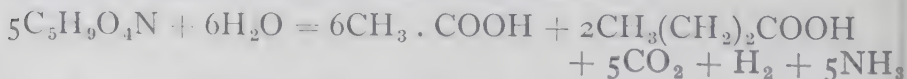
If no hydrogen donator is added to the system the organism, if present in sufficient amount, effects the reduction at the expense of the endogenous reducing materials of the cell. It is unknown whether this effect is produced by one enzyme or two, though in the case of the reductive deamination of aspartic to succinic the effect may be produced by the combined action of aspartase followed by succinic dehydrogenase. Reductive deamination can be demonstrated clearly when the organism contains hydrogenase and H_2 acts as the donator; the reductive deamination of glycine, tryptophan and ornithine has been shown in this way.²

4. Hydrolytic deamination

Deamination by intact cells frequently gives rise to the corresponding α -hydroxy acid. This dual action may possibly in some cases be the work of one enzyme as in the hydrolytic deamination of aspartate to malate,³ but more probably, as in the case of the aspartic-fumaric-malic cycle, it is brought about by a desaturation followed by hydrolysis.

5. Anaerobic decomposition with evolution of hydrogen

A recently described decomposition of amino-acids is an anaerobic oxidative deamination accompanied by evolution of hydrogen. This has so far only been reported for seven amino-acids, using *Cl. tetanomorphum* in washed suspensions. In the case of glutamic acid the products of decomposition have been quantitatively determined and agree with the following equation:⁴



This type of reaction may perhaps be regarded as an anaerobic

¹ Gale, 1938.

³ Virtanen & Erkama, 1938.

² Hoogerheide & Kocholaty, 1938.

⁴ Woods & Clifton, 1937, 1938.

device for obtaining energy from amino-acids without the use of a hydrogen acceptor. The immediate precursor of hydrogen is not known, but is not formate, since *Cl. tetanomorphum* has no formic hydrogenlyase.¹

6. Mutual oxidation and reduction by pairs of amino-acids

Amongst the proteolytic anaerobes the energy for cell synthesis must be derived from proteins or their digestion products. It therefore seems reasonable to look to the decomposition of amino-acids for reactions likely to furnish this energy, and since oxidation by molecular oxygen can play no part, mutual oxidation and reduction appears a probable course. By the use of washed suspensions of *Cl. sporogenes* and the methylene blue technique, it has been shown that the following amino-acids are oxidisable,^{2,3} i.e. serve as hydrogen donators: *L*(-)-alanine, *L*(-)-valine, *L*(-)-leucine, *L*(-)-phenylalanine, etc. Furthermore, by the technique described on p. 47, it can also be shown that the following are reducible, i.e. serve as hydrogen acceptors: glycine, *L*(-)-proline, *L*(-)-hydroxyproline, *L*(+)-ornithine and *L*(+)-arginine. Moreover, when one amino-acid from either group is incubated *in vacuo* with the organism, no deamination or only a very slow one occurs, but when a donator and an acceptor are present together, we get deamination and decomposition of both donator and acceptor, the former becoming oxidised and the latter reduced. The following experiment makes this clear:

In a Thunberg tube were placed 0.5 ml. *M*/10 solutions of the donator and the acceptor; three similar control tubes were also set up, the first containing the donator alone, the second the acceptor alone and the third neither amino-acid; all tubes contained buffer and bacterial suspensions. The tubes were evacuated and incubated at 40° C. for 2-6 hours, after which the free ammonia was estimated in each tube. The results are seen in Table 2.

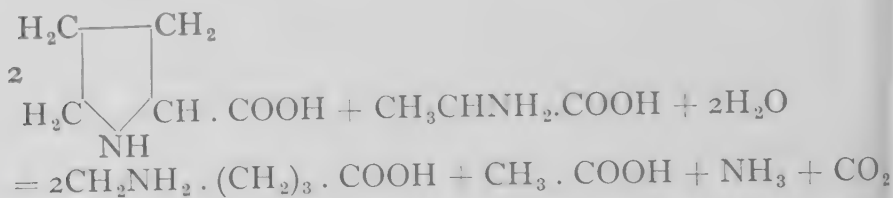
TABLE 2

	Ammonia found, ml. N/10	Ammonia (cor- rected for blank), ml. N/10
Blank	0.07	—
0.5 ml. <i>M</i> /10 <i>L</i> (+)-alanine	0.06	0.00
0.5 ml. <i>M</i> /10 <i>L</i> (-)-proline	0.05	0.00
0.5 ml. alanine + 0.5 ml. proline	0.42	0.35

¹ Wood & Cullen, 1937.² Suckland, 1934.³ Woods, 1936 (a).

Moreover, any one of the hydrogen donors will react with any one of the hydrogen acceptors, and the reaction proceeds at a rate comparable with essential processes such as aerobic oxidations occurring in other bacteria with a similar growth rate. For this purpose the $Q_{O_2}^{\text{alanine}}$ of *Cl. sporogenes* was calculated and found to vary from 5 to 12; this is undoubtedly low owing to the instability of the enzymes concerned in washed suspensions, but is of the same order as that measured for the aerobic oxidation of lactate by *Bact. coli*.

The products of oxidation and reduction of donors and acceptors respectively have been ascertained quantitatively in some cases. It was first shown that *l*-proline is reduced by *l*-alanine to δ -amino-*N*-valeric acid;¹ i.e. the ring is split but no deamination occurs. It was next found that in the same reaction alanine is oxidised to acetic acid, ammonia and carbon dioxide, probably with the intermediate production of pyruvic acid. The reaction is thus represented:



Glycine, when acting as hydrogen acceptor, is reduced thus:²

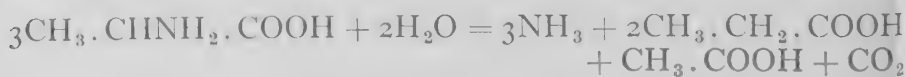


Ornithine, when acting as hydrogen acceptor, behaves like *l*-proline, i.e. is reduced to δ -amino-*N*-valeric acid.³

It is interesting to note that all the amino-acids taking part in the above reactions occur in the synthetic medium for *Cl. sporogenes*.⁴

7. Decomposition by mutase reaction

Closely allied to the oxidoreductions just described is the decomposition of an amino-acid in which one molecule is oxidised whilst the other is reduced. Thus alanine is decomposed according to the equation:



This may proceed as a mutase reaction in which 2 mols. of alanine are reduced at the expense of the oxidation of 1 mol. to acetic acid

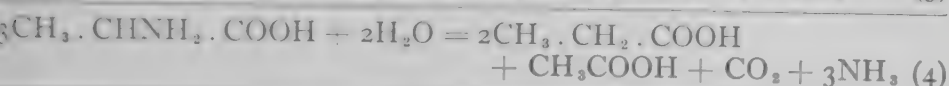
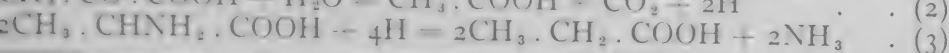
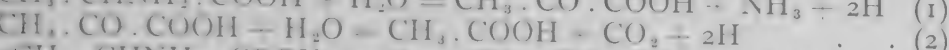
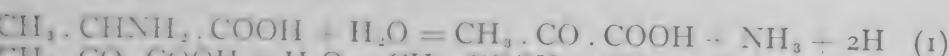
¹ Stickland, 1935 (1).

² Ibid., (2).

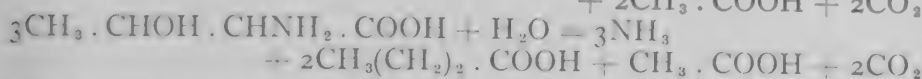
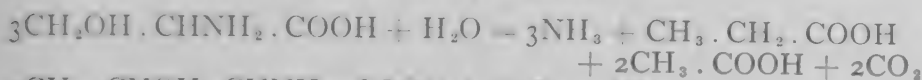
³ Woods, 1936 (2).

⁴ Fildes & Richardson, 1935.

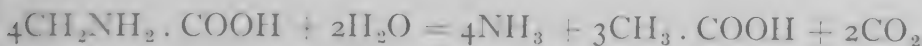
and CO_2 . This reaction appears to be analogous to the fermentation of lactate by propionic bacteria.



Serine and threonine are similarly dismuted.



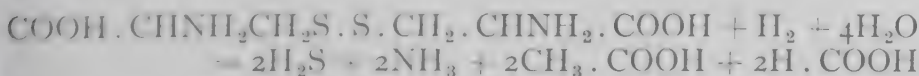
Lactic and pyruvic acids are fermented as in propionic fermentation. The organism responsible is a hitherto undescribed *Clostridium*. A gram-negative *coccus* decomposes glycine in a similar way:



Under certain conditions 0.35 mol. of H_2 may be given off per mol. of glycine whilst the acetic acid is decreased and the CO_2 increases, some of the hydrogen being liberated instead of being used for reduction.¹

Sulphur-containing amino-acids

Cystine is decomposed after preliminary reduction to cysteine. This may be effected by hydrogen (in the presence of hydrogenase), glucose or other reducing agents; H_2S and NH_3 are liberated in equimolecular proportions.² Using *Proteus vulgaris* the decomposition agrees with the equation:



In the case of *Bact. coli* and *P. vulgaris* the enzyme liberating H_2S (cystinase) is adaptive, attacks only the *L*-isomer and is somewhat inhibited by glucose. In the case of *Propionibacterium pentose-accum* the enzyme is constitutive and accelerated by glucose.^{3, 4, 5}

The decomposition of tryptophan

Owing to the physiological importance of this amino-acid its decomposition by micro-organisms has always excited much interest. In 1875 Kühne obtained indole by the combined action of

¹ Cardon, 1942, and Cardon & Barker, 1947.

² Tarr, 1933.

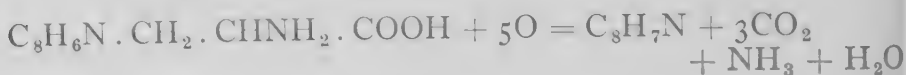
³ Deamuelle & Fromageot, 1939.

⁴ Deamuelle, 1939.

⁵ Ibid., 1940.

pancreas and of putrefactive organisms on meat;¹ later indole and indoleacetic acid were repeatedly identified.² The precursor of these compounds remained unknown till 1903 when tryptophan was isolated by Hopkins and Cole.³ Its discoverers immediately saw in this amino-acid the probable precursor of the indole of putrefaction and demonstrated the production of indole and β -indoleacetic acid by the aerobic growth of *Bact. coli* on a medium of pure tryptophan and salts to which gelatin (which contains no tryptophan) was added later to improve growth. In anaerobic conditions β -indolepropionic acid was produced both by *Bact. coli* and by Rauschbrand's bacillus. This is one of the earliest examples in bacterial chemistry of the use of pure strains of bacteria on chemically defined media.

Amongst indole-producing organisms *Bact. coli* has been the most extensively studied. Using growth experiments in pure tryptophan and salts in which the amino-acid supplied the sole source of nitrogen, Herzfeldt and Klinger⁴ proved a stoichiometric relationship between the indole formed and the tryptophan disappearing. Woods,⁵ using thick washed suspensions of *Bact. coli* in phosphate buffer continuously aerated, showed that *l*-($-$)tryptophan is converted quantitatively to indole, and that the rate of disappearance of tryptophan is identical with the rate of production of indole, and furthermore that the oxygen taken up corresponds with the complete oxidation of the side chain of tryptophan to carbon dioxide and water:



The unnatural isomer *d*-($+$)tryptophan is attacked only very slowly or not at all. The same author showed that in similar conditions anaerobically *Bact. coli* converts tryptophan to β -indolepropionic acid, the hydrogen necessary for this reaction coming presumably from endogenous hydrogen donors of the cell. These results have been confirmed by Majima.⁶

A non-viable preparation from *Bact. coli* ("tryptophanase") has been obtained by Happold and Hoyle⁷ by treating washed suspensions with chloroform, and by precipitation with alcohol and desiccation with alcohol and ether. This preparation produces indole from tryptophan under favourable conditions of aeration in yields of 80-90%. The potency of the preparation was increased if the organisms were grown on a medium rich in tryptophan. Later an active cell-free preparation was obtained by extracting an

¹ Kühne, 1875.

² Salkowski & Salkowski, 1879, 1880, 1885.

³ Hopkins & Cole, 1901, 1903.

⁴ Herzfeldt & Klinger, 1915.

⁵ Woods, 1935 (1).

⁶ Majima, 1936.

⁷ Happold & Hoyle, 1935.

acetone powder prepared from the cells with borate buffer pH 8-8.5.¹ It was also found that strains of *Bact. coli* which were poor indole formers rapidly improved if subcultivated on a medium containing sodium desoxycholate (1 in 1000); this salt has no effect on the production of indole by the tryptophanase preparation.

The actual course of the change of tryptophan to indole has been much studied. In growth experiments Frierer² found no production of indole from the following compounds: α -indolecarboxylic acid, α - β -indoledicarboxylic acid, β -indolepyruvic acid, β -indoleethylamine. Saito,³ in growth experiments, showed qualitatively some indole formation from *dl*- β -indolelactic and β -indolepyruvic acid, provided that an alternative source of nitrogen was present. The subject has also been studied by Woods,⁴ using thick bacterial suspensions. In conditions in which 100% of indole was obtained from tryptophan no indole was produced from β -indolealdehyde, β -indolecarboxylic acid, β -indoleacetic acid, β -indolepropionic acid and β -indoleacrylic acid. In the case of β -indolepyruvic acid a 10% production of indole was found, but only in the presence of ammonium salts (or of tryptophan). The small formation of indole (as compared with that from tryptophan) and the necessity for ammonia make it unlikely that β -indolepyruvic acid is the true intermediate. Woods suggests that β -indolepyruvic acid and ammonia are first synthesised to tryptophan and that this subsequently yields indole by the usual (but still unknown) route. This observation has been confirmed by Majima, who also found that *d*-(-)- β -indolelactic acid gives indole when aerated with thick suspensions of *Bact. coli*, but considers that the smaller rate of formation and lower yield (as compared with tryptophan) make it unlikely that it is a true intermediate. Finally Happold and Hoyle, using their tryptophanase preparation, found no production of indole from β -indolealdehyde, β -indolepropionic acid, β -indoleacetic acid, β -indoleacrylic acid and β -indolepyruvic acid.

Most of the routes suggested at various times for the production of indole from tryptophan are in contradiction to the above results; among them may be cited the following:

1. $R \cdot CH_2 \cdot CHNH_2 \cdot COOH \rightarrow R \cdot CH_2 \cdot CH_2NH_2 + CO_2$
2. $R \cdot CH_2 \cdot CHNH_2 \cdot COOH \rightarrow R \cdot CH_2 \cdot CO \cdot COOH + NH_3$
3. $R \cdot CH_2 \cdot CHNH_2 \cdot COOH \rightarrow R \cdot CH:CH \cdot COOH + NH_3$
4. $R \cdot CH_2 \cdot CHNH_2 \cdot COOH \rightarrow R \cdot CH_2 \cdot CHOH \cdot COOH + NH_3$

It is unlikely that any of these reactions occur as a preliminary to indole production since the indole derivatives so formed are either incapable of forming indole (1 and 3) or do so at a slower rate and

¹ Dawson, *et al.*, 1946. ² Frierer, 1921. ³ Saito, 1933. ⁴ Woods, 1935 (2).

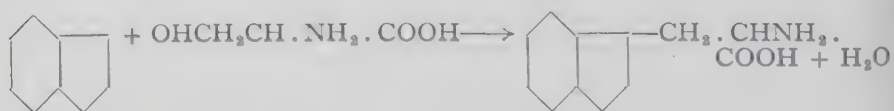
less completely than tryptophan (4), or do so only in special conditions (2).

A number of derivatives of tryptophan in which the side chain was modified by the introduction of substituting groups failed to produce indole;^{1, 2} this led Baker and Happold to the conclusion that the breakdown requires a free carboxyl and an unsubstituted α -amino group and a β -carbon susceptible to oxidative attack.

It has been shown that *Bact. coli* oxidises *o*-amino- β -phenyl-ethanol with the production of indole at a rate three times as great as that by which it produces indole from tryptophan. The possibility therefore exists that the former compound may be an intermediary in the formation of indole and a scheme has been presented in accordance with this hypothesis.³

A new type of evidence has been supplied by work on the mould *Neurospora*. Two mutant strains of this organism have been obtained, both unable to grow without tryptophan. In the one tryptophan can be replaced by indole, in the other by either indole or anthranilic acid. Other indole derivatives and kynurenine were negative. When indole was used its disappearance was accelerated by the addition of *l*(-)-serine (alanine did not replace serine). When serine is the limiting factor for growth the rate of disappearance of indole is a function of the serine concentration.

Moreover, when dried mycelia were shaken with 50 mg. indole and 500 mg. serine all the indole disappeared in 48 hours and *l*(-)-tryptophan was found in the medium, isolated and identified. The authors state also that *Bact. coli* synthesises tryptophan from indole and serine and suggest that the synthesis of tryptophan occurs thus:⁴



The breakdown of tryptophan by the reverse process would account for the oxygen uptake (5 atoms oxygen per mol. indole) found by Woods. A breakdown in the presence of mepacrine to indole and alanine has been reported.⁵

The bacterial attack on the indole ring was early demonstrated; for example, *B. pyocyaneus* and *B. fluorescens* attack both the side chain and the ring of tryptophan,^{6, 7} whilst *Proteus vulgaris* and *Bact. coli* decompose only the side chain.^{8, 9}

A different type of indole oxidation has been reported by

¹ Baker & Happold, 1940.

³ Krebs *et al.*, 1942.

⁴ Tatum & Bonner, 1944.

² Fellers & Clough, 1925.

⁵ Dawes *et al.*, 1946.

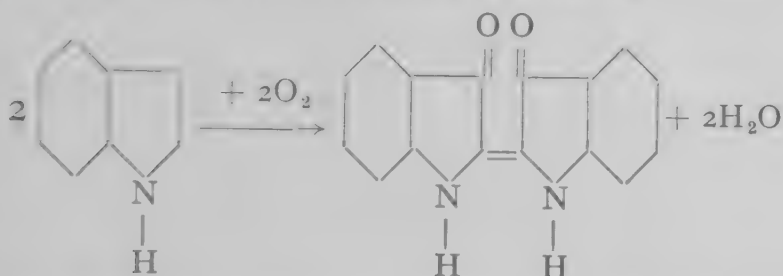
⁶ Raistrick & Clark, 1921.

⁷ Supniewski, 1924.

⁸ Herzfeldt & Klinger, 1915.

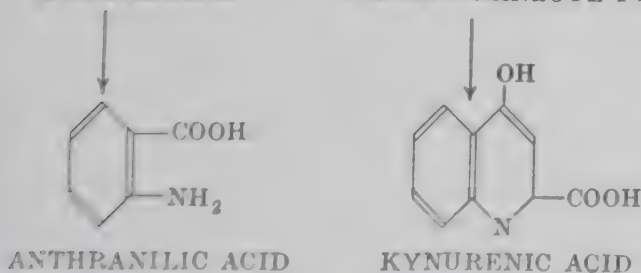
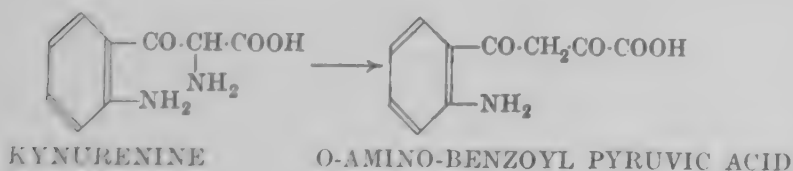
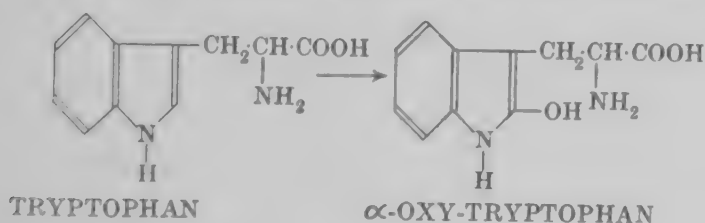
⁹ Woods, 1935 (1).

Gray;¹ two species isolated from soil (*Ps. indoloxidans* and *Mycobact. glaberulum*) oxidise indole to indigotin.



These organisms are unable to produce indole from tryptophan.

Yet another type of rupture of the ring was shown by Kotake to occur through the action of *Bac. subtilis*. From tryptophan in the presence of glycerol it was possible to isolate kynurenic acid and anthranilic acid;² kynurenic acid and anthranilic acid could also be isolated when kynurenine was substituted for tryptophan, hence Kotake postulated the breakdown of tryptophan in this system as occurring according to the following scheme:



Later work³ showed that Kotake's formula for kynurenine is wrong and the formula more recently established has been substituted in the above scheme.

¹ Gray, 1928.

² Kotake, 1933.

³ Butenandt *et al.*, 1943.

In the formation of kynurenic acid therefore we have a primary disruption of the indole nucleus followed by a stabilisation to a different type of ring. Such a disruption might also occur with *Bact. coli* followed by ring closure re-forming the indole nucleus. The action of *coli* on kynurenine has not been reported; Woods found no formation of indole from kynurenic acid.¹

The intracellular synthesis of tryptophan

Some insight into the intracellular synthesis of tryptophan has been furnished by the use of organisms which have lost one or more of their synthetic powers and as a consequence require to be supplied with a compound which previously they were able to synthesise. Such organisms have received attention from Fildes working with naturally occurring mutants of bacteria,² and by Tatum and Bonner³ working with artificially induced mutants of *Neurospora crassa*. Many strains of *Bact. typhosum* are exacting towards tryptophan and it has been shown that in the case of four such exacting strains as well as of three strains of *C. diphtheriae* tryptophan can be replaced by indole though 10 to 100 times as much of the latter is required. Some have been trained to dispense with it. An exacting strain of *Staphylococcus*, however, could not tolerate this replacement. Similar results were obtained with certain tryptophan-exacting strains of lactic bacteria, five of which could replace tryptophan by indole or anthranilic acid.

Turning to *Neurospora crassa*, the wild strain A synthesises tryptophan on synthetic medium (nitrogen being supplied by ammonia); mutants 1 and 2 require to have it supplied. Mutant 1 accepts either anthranilic acid or indole in place of tryptophan, as do the two lactic⁴ organisms; mutant 2 accepts only indole. Three hitherto unknown enzymes or mechanisms are thus revealed: (1) concerned in the production of anthranilic acid from some unknown precursor; (2) concerned in some stage from anthranilic acid to indole; and (3) catalysing the condensation of indole with l-serine to give tryptophan (see Fig. 4).

The first group of deficient organisms, D and E, has lost 1, but retains 2 and 3; the second group, F and G, has lost 1 and 2; *Staphylococcus* has lost 1, 2 and 3.

Further information has been gained by the use of a stereo-inhibitor indoleacrylate.⁵ This has been rigidly proved by Fildes not to give indole by any of the bacterial strains used. Growing cultures of B, C and F in the presence of indoleacrylate now all produce indole, B and C in the absence of tryptophan in the medium and F in excess of the tryptophan supplied; the poison has

¹ Unpublished observation.

³ Tatum & Bonner, 1944.

⁴ Snell, 1943.

² Fildes, 1941, 1945.

⁵ Fildes, 1941, 1945.

blocked the third step; indole no longer passes to tryptophan at the optimal rate and so accumulates in the medium. We have here the surprising phenomenon of *Bact. typhosum* producing indole (but not from tryptophan), indoleacrylate producing this change by stereo-inhibition of enzyme 3 (see Fig. 4).

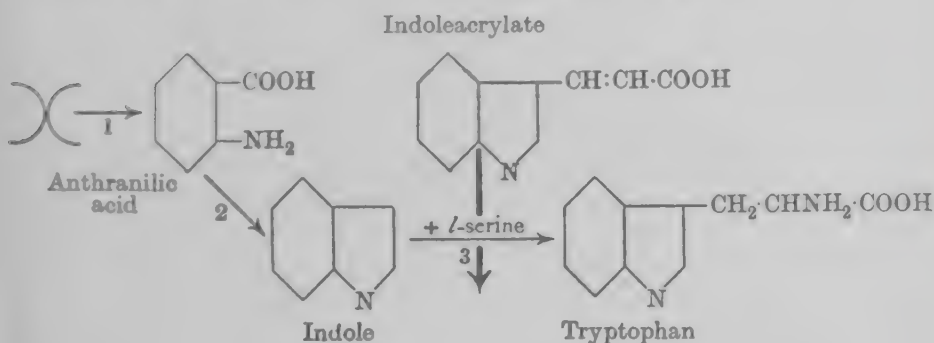


FIG. 4

Organism	Mechanisms present		
A. <i>Neurospora</i> (wild strain)	.	.	1 2 3
B. <i>Bact. coli</i>	.	.	1 2 3
C. <i>Bact. typhosum</i> (non-exacting)	.	.	1 2 3
D. Lactic organisms (two strains)	.	.	2 3
E. <i>Neurospora</i> (mutant 1)	.	.	2 3
F. <i>Bact. typhosum</i> (exacting strains)	.	.	3
G. <i>Neurospora</i> (mutant 2)	.	.	3
H. <i>Staphylococcus</i>	.	.	- - -

The production of skatole

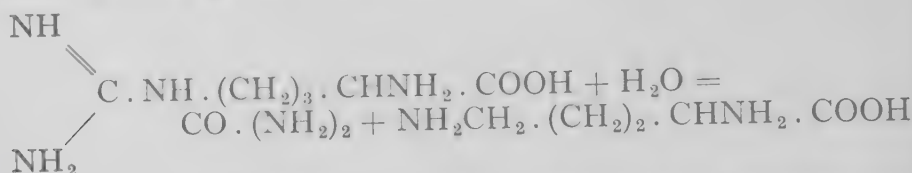
The isolation of skatole has often been reported from mixed putrefactions and from faeces. The isolation has, however, usually involved the steam distillation from acid (and from alkali) solution. Frierber¹ has shown that when β -indoleacetic acid is steam-distilled from acid solution it is decarboxylated, producing skatole. Since β -indoleacetic acid is a common bacterial decomposition product of tryptophan it is apparent that the skatole isolated may often have been an artefact. The only well-authenticated case of skatole production is that by *Cl. skatole* anaerobically from peptone by Fellers and Clough.²

The decomposition of arginine

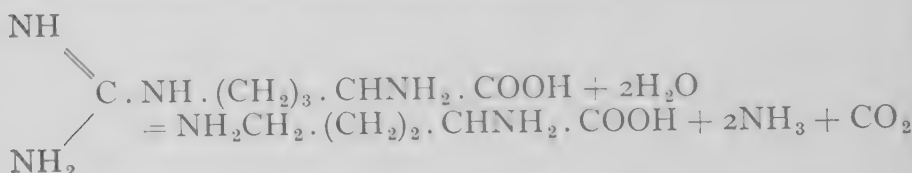
The decomposition of arginine calls for special notice. The production of ornithine was early observed^{3, 4} as a result of the action of mixed putrefactive organisms. It was assumed that this

¹ Frierber, 1921.² Ackermann, 1908.³ Fellers & Clough, 1925.⁴ Ellinger, 1899.

was due to the enzyme arginase known in animal tissues which decomposes arginine to urea and ornithine :



A notable paper by Hills,¹ however, has altered the interpretation of the evidence. Hills, studying the gram-positive *coccaceæ*, found that this group showed practically no ammonia production on any amino-acid except arginine. This compound was not in fact deaminated but decomposed according to the equation

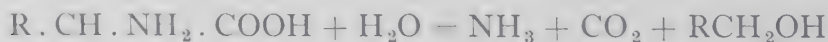


The possibility of the reaction being due to arginase in conjunction with urease was ruled out by the fact that the latter enzyme was absent from all the seven strains of streptococci examined, though present in the staphylococci; in the latter, however, the rate of NH_3 production from urea was less than that from arginine. The ornithine formed is not further deaminated at either the α - or δ -linkage by this group of bacteria, though its reductive deamination to δ -aminovaleric acid by *Cl. sporogenes* has been shown.²

Various factors profoundly affect the deaminative power of bacteria, most particularly the presence of carbohydrate and the reaction of the media during growth. These will be dealt with in Chapter XI. It may be stated here, however, that the presence of fermentable carbohydrate in the medium inhibits the formation of deaminases apart altogether from any effect due to pH.

8. Hydrolytic deamination and decarboxylation

The action of yeasts, so far as is known at present, is to attack amino-acids by a combination of deamination, decarboxylation and hydrolysis.

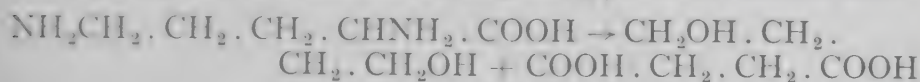


The study of this mechanism is due to the classical investigations of Ehrlich and little work on the action of yeasts on amino-acids has been reported since his time. Amino-acids shown to be at-

¹ Hills, 1940.

² Woods, 1936 (2).

tacked in this way are histidine,¹ phenylalanine,² tryptophan,³ tyrosine;⁴ the conditions of the reaction were the presence of large quantities of yeast, nutritive salts, including ammonia, and the simultaneous fermentation of glucose. More recently Thorne has shown that the same reaction occurs in conditions similar to those in the brewer's vat; glutamic acid and valine underwent the type reaction; arginine was first decomposed to urea and ornithine, the latter giving rise to a mixture of butylene glycol and succinic acid.⁵



9. Decarboxylation

The presence of amines due to bacterial action on amino-acids has been sporadically reported at intervals, first by mixed putrefactive bacteria and later by pure cultures. The observations of Koessler and Hanke⁶ and of Eggerth (who all worked with growing organisms) suggested that a prerequisite for amine production might be an acid medium and a low temperature of incubation.

Following these clues Gale made a comprehensive study of the conditions governing amine production by bacteria and these are now as clearly defined as in the case of deamination. The principal factor in the production of the decarboxylases is a low pH during growth, i.e. 5.0 or lower. This is most readily achieved by the addition of 2% glucose to the medium. In the case of coliform organisms a low growth temperature (25–28°) is also important, the enzymes not being fully active till the end of the growth period.⁹

In the course of these studies decarboxylases for six amino-acids only have been found, viz. *l*(+)-lysine, *l*(+)-arginine, *l*(+)-ornithine, *l*(-)-glutamic acid, *l*(-)-histidine and *l*(-)-tyrosine;¹⁰ tryptophan decarboxylase was reported earlier but has not been encountered in these recent studies.¹¹

Decarboxylases are not as widely distributed amongst bacteria as are deaminases; for example, out of 151 strains of *Esch. coli* examined 114 possessed decarboxylase for arginine, 142 for lysine, 130 for glutamic acid and 14 for histidine. Amongst the streptococci decarboxylase activity seems limited to tyrosine and this occurs chiefly in Lancefield's group D and less often in A.¹² Among the *Clostridia* decarboxylases for histidine, glutamic acid and tyrosine have been found,¹³ weak decarboxylases for arginine,

^{1,2,3,4} Ehrlich, 1910, 1907 (1), 1912, 1911.

⁵ Thorne, 1937.

⁶ Koessler & Hanke, 1919.

⁷ Hanke & Koessler, 1922, 1924.

⁸ Eggerth, 1939.

⁹ Gale, 1940 (1), (2), (3), (4); 1941 (1).

¹⁰ Ibid.

¹¹ Berthelot & Bertrand, 1912.

¹² Gale, 1940 (2).

¹³ Ibid., 1941 (1).

lysine, ornithine and glutamic acid among the *Bacillaceae*, and for glutamic acid and ornithine¹ in *Proteus vulgaris* and *morganii*.² Among groups where decarboxylases occur many strains possess none, and the distribution of those present seems completely haphazard. Besides a low growth pH and temperature the presence of the amino-acid in the medium enhances the production of the enzyme. The *l*-series of amino-acids only is attacked.

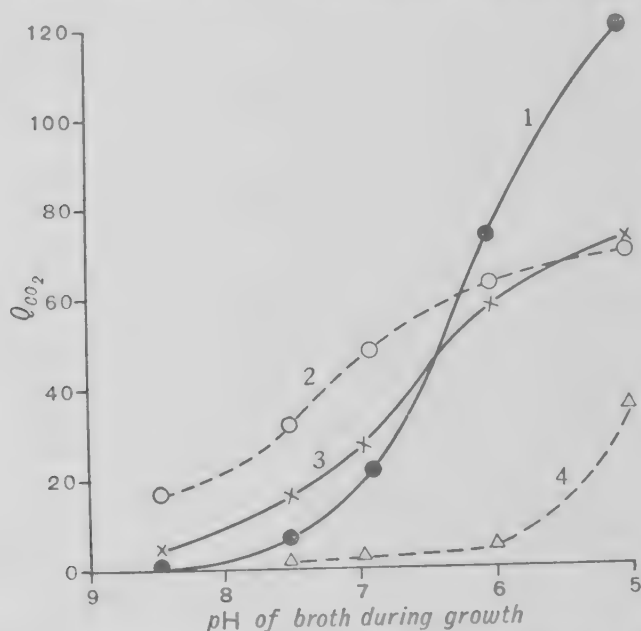


FIG. 5.—Variation of decarboxylase activity of washed suspensions of *Bact. coli* (stock) with the pH of the plain broth in which the organism was grown. 1. Arginine. 2. Glutamic acid. 3. Lysine. 4. Histidine³

By the use of manometers it can be shown that the amino-acids in question are quantitatively decarboxylated to the corresponding amines which, by working on a larger scale, can be isolated and identified as picrates or, in the case of γ -aminobutyric acid, as the Ag salt.

Table 3⁴ summarises the distribution of the decarboxylases in studies by Gale *et al.*

All six amino-acid decarboxylases have been obtained in a cell-free condition. Except in the case of tyrosine decarboxylase this has been achieved by extracting the acetone-treated cells with borate buffer at pH 8.5; in the case of tyrosine decarboxylase acetate buffer at pH 5.5 was used. Further purification is carried

¹ Gale, 1946.

² Ibid., 1941 (1).

³ Ibid., "The Production of Amines by Bacteria," 1940, *Biochem. J.*, **34**, 400.

⁴ Ibid., 1946.

out by adsorption and elution followed by fractional precipitation with ammonium sulphate. The ornithine enzyme is labile to acetone, and cell-free preparations are prepared by cell disintegration followed by centrifugation.^{1, 2, 3, 4, 5} The activity thus reached

TABLE 3

Organism	Enzyme substrate					
	Arginine	Ornithine	Histidine	Lysine	Tyrosine	Glutamic acid
<i>Esch. coli</i>	+	+	+	+	+	+
<i>Klebs. pneumoniae</i>	—	+	+	+	—	—
<i>Str. faecalis</i>	—	—	—	—	+	—
<i>Proteus vulgaris</i>	—	+	—	—	—	+
<i>Proteus morganii</i>	—	+	—	—	—	+
<i>Cl. welchii</i>	—	—	+	—	—	+
<i>Cl. sporogenes</i>	—	—	—	—	—	—
<i>Cl. septicum</i>	—	+	—	—	—	—
<i>Cl. aerofætidum</i>	—	—	—	—	+	+

measured as Q_{40}^0 at 30° was 46,000 for lysine decarboxylase, 46,300 for tyrosine decarboxylase and 3670 for histidine decarboxylase.⁶ If the necessary precautions are taken each of the six purified enzymes is specific for one amino-acid and can be used for its assay in protein hydrolysates and for the resolution of racemic mixtures.⁷ The pH optima lie in a narrow range between 2.5 and 6.0; in the case of the histidine, lysine and arginine enzymes the pH optimum of the purified enzymes is 1.5–2.0 pH units higher than that measured in cell suspensions. The properties of the decarboxylases in the intact cell and in cell-free preparations are compared in Table 4.

TABLE 4⁸

PROPERTIES OF THE AMINO-ACID DECARBOXYLASES

Decarboxylase	pH optimum		Michaelis Constant	
	Intact cell preparation	Cell-free preparation	Intact cell preparation	Cell-free preparation
<i>l</i> (+)-Histidine	2.5–3.0	4.5	0.00075 <i>M</i>	0.00075 <i>M</i>
<i>l</i> (+)-Lysine	4.5–5.0	6.0	0.00280 <i>M</i>	0.00150 <i>M</i>
<i>l</i> (+)-Arginine	4.0–4.8	5.2	0.00056 <i>M</i>	0.00075 <i>M</i>
<i>l</i> (+)-Glutamic acid	4.0–4.5	4.5	0.005 <i>M</i>	0.027 <i>M</i>
<i>l</i> (+)-Ornithine	5.0–5.5	5.2	0.003 <i>M</i>	0.004 <i>M</i>
<i>l</i> (+)-Tyrosine	5.0–5.5	5.5	—	—

The cell-free decarboxylases for lysine, arginine, ornithine and tyrosine can each be resolved into an apoenzyme and coenzyme

¹ Gale, 1944 (1).² Epps, 1944.³ Gale & Epps, 1944 (1).⁴ Epps, 1945.⁵ Taylor & Gale, 1945.⁶ Gale, 1946.⁷ Ibid.⁸ Ibid.

by precipitation with alkaline ammonium sulphate.¹ The ornithine enzyme undergoes spontaneous resolution on standing; the glutamic enzyme has been resolved by dialysis at pH 2.0.² The apoenzyme so obtained is without activity until some preparation of codecarboxylase is added. This can be supplied by boiled extracts of bacteria or by boiled preparation of the purified enzyme before treatment, and the activity is then proportional over a narrow range to the amount of coenzyme added; the apoenzyme can therefore be used as a test material for the coenzyme. The latter has a very wide distribution, being found in all bacteria so far tested (including those containing no decarboxylases) and in many animal and in plant tissues.³ It has been prepared in a highly concentrated condition from yeast as the lead salt and the apoenzyme is then half-saturated with respect to the coenzyme preparation when the latter reaches a concentration containing 0.06 $\mu\text{g. carbon/ml.}$ ⁴

The enzyme concentrate thus prepared is active with the five decarboxylases mentioned above. So far the decarboxylase for histidine has not been resolved into apo- and coenzyme, nor do purified preparations of this enzyme, when boiled, yield solutions which activate the apoenzymes of the other four. The codecarboxylase is not replaceable by any known coenzyme or growth factor, not excluding pyridoxine. The further elucidation of its nature is due to an ingenious biological experiment of Bellamy and Gunsalus.⁵ These investigators made use of a strain of *Str. faecalis* which on a medium containing optimal amounts of all the necessary growth factors produces tyrosine decarboxylase but on a restricted medium, even in the presence of tyrosine, has negligible activity. It was further shown that when a strain of *Str. faecalis* R, which is generally exacting towards pyridoxine, is grown in the presence of alanine it can dispense with the former compound but then produces no tyrosine decarboxylase; the cells can, however, then be activated by the addition of pyridoxal, the aldehyde derivative of pyridoxine. Using washed suspensions of these pyridoxine-deficient streptococci the apoenzyme is half-saturated with respect to pyridoxal at 0.05 $\mu\text{g./ml.}$ It appears, however, that pyridoxal is not the true coenzyme as it is inactive with dried cells and with cell-free preparations. If, however, the pyridoxal is phosphorylated or used in conjunction with a phosphorylating agent (A.T.P.) it is active with apoenzyme preparations of tyrosine, lysine, arginine and ornithine decarboxylases.⁶ It is probable that the codecarboxylase prepared from yeast is

¹ Gale & Epps, 1944 (1).

³ Gale & Epps, 1944 (1).

⁵ Bellamy & Gunsalus, 1944 (1), (2).

⁶ Gunsalus, Bellamy & Umbreit, 1944.

² Umbreit & Gunsalus, 1945.

⁴ Epps, 1944.

identical with pyridoxal phosphate, judging by stability measurements and the identical action of the two compounds on the rate of evolution of CO_2 by the five apodecarboxylases¹ (see Table 5 and Fig. 6).

TABLE 5
CODECARBOXYLASE ACTIVITY OF PYRIDOXAL PHOSPHATE

	$\mu\text{l CO}_2$ liberated from substrate per 5 min.		
	Alone	+ Codecarboxy- lase	+ Pyridoxal phosphate
<i>l</i> (-)-Tyrosine . . .	2	110	116
<i>l</i> (+)-Lysine . . .	15	106	104
<i>l</i> (+)-Arginine . . .	15	70	75
<i>l</i> (+)-Ornithine . . .	29	78	74

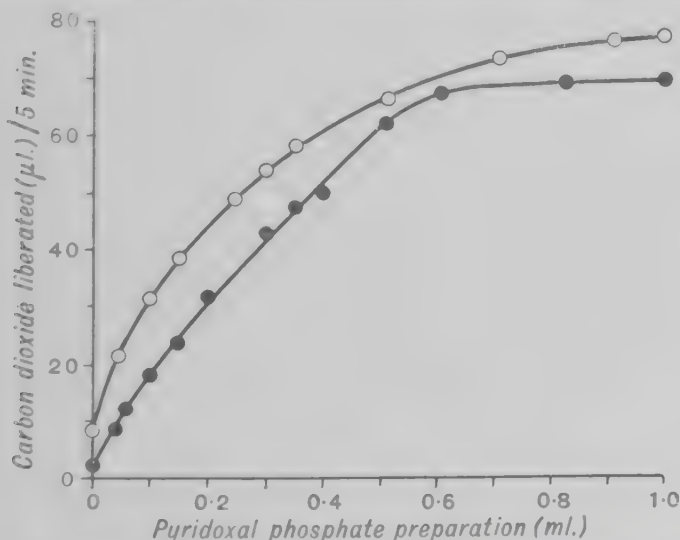
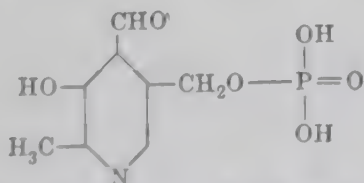


FIG. 6.—Effect of “pyridoxal phosphate” concentration on rate of decarboxylation of *l*(-)-tyrosine by tyrosine apodecarboxylase (●—●); of *l*(+)-lysine by lysine apocarboxylase (o—o)²

The position of the phosphate radicle is not certain; Karrer and Viscontini claim that it is on position 5, a finding with which Gunsalus and Umbreit disagree; position 3 seems the most probable alternative.^{3, 4, 5, 6}



¹ Boldly & Gale, 1945.

² Gunsalus *et al.*, 1945.

³ Karrer & Viscontini, 1947.

⁴ *Ibid.*, *Nature*, 155, 728 (1945).

⁵ Harris *et al.*, 1944.

⁶ Gunsalus & Umbreit, 1947.

Functions of deaminases and decarboxylases

One of the functions of bacterial deaminases is probably to liberate ammonia for growth from proteins, or rather from their decomposition products. The corresponding function of the decarboxylases is more difficult to understand. CO_2 is necessary for bacterial growth, but one would imagine that in natural environments this would be present in ample supply from other sources. At reactions below $\text{pH } 5.5$, where decarboxylases are active, the solubility of CO_2 is extremely low and an endogenous supply may then become important. It is noteworthy that deaminases and decarboxylases are formed and active at alkaline and acid reactions

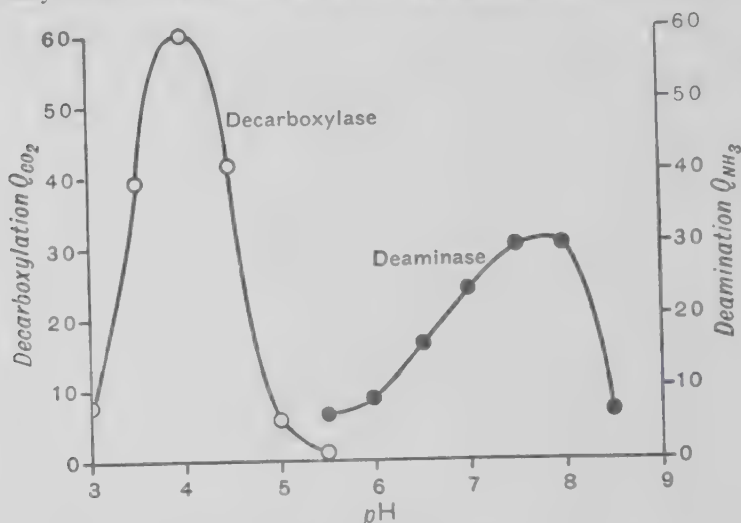


FIG. 7.—Variation with pH of the activities of the enzymes of *Esch. coli* which attack *l*-glutamic acid²

respectively, and that there exists no value of pH at which both are simultaneously active¹ (Fig. 7). This suggests that for deamination and decarboxylation to occur it is necessary that the $-\text{COOH}$ and $-\text{NH}_2$ groups respectively should be undissociated; furthermore it seems that the undissociated groups only are capable of influencing the modification of the developing bacterial proteins to form decarboxylases and deaminases. In the case of the decarboxylases it is noteworthy that they are formed only for amino acids with a strong polar group at the end furthest removed from the carboxyl, though all amino-acids with such a configuration have not so far been found to give rise to decarboxylases.

10. Transamination

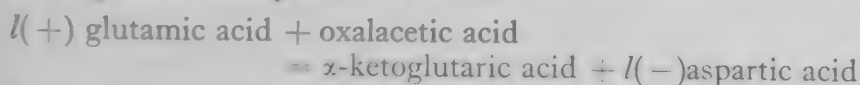
The transamination reaction was first shown by Braunstein and

¹ Gale, 1940 (1).

² Ibid., *Bact. Rev.* (1940), 4, 165.

Kritzmann¹ in muscle. It consists of the transfer of an $-\text{NH}_2$ group from either aspartic or glutamic acid to the α -position in an α -keto-acid, and it has been suggested that such a transfer is important in the general synthesis of amino-acids.

The reaction was first demonstrated for bacteria by Lichstein and Cohen,² who showed that suspensions of *coli* and a number of other organisms catalysed the reaction :



Previous attempts to demonstrate this reaction had failed since the aspartic acid is removed very rapidly by various side-reactions and can be estimated only if very short incubation periods are used. The rate of reaction is optimal at pH 8.5 and 32°. The rate of transamination is measured as Q_{TN} ($\mu\text{l.}$ aspartic acid formed/mg. cell N/hr.) and values for a representative collection of organisms are given in Table 6.

TABLE 6

Organism	Activity (Q_{TN})
<i>Bac. coli</i>	890
„ <i>dysenteriae</i> (Shiga)	685
„ <i>typhosus</i>	1135
„ <i>proteus</i>	1610
„ <i>pyocyaneus</i>	800
<i>Azotobacter vinelandii</i>	1517
<i>Staph. aureus</i>	910
„ <i>albus</i>	950
<i>Bac. welchii</i>	1170
<i>Str. haemolyticus</i>	865
<i>Str. viridans</i>	900
<i>Pneumococcus</i> (type I)	845

Lichstein *et al.*³ have obtained a cell-free preparation from *Str. faecalis* which will catalyse the transamination reactions. They showed by two methods that the prosthetic group of the enzyme is pyridoxal phosphate: first, if the organism is grown in a medium deficient in pyridoxin, then the transaminase activity of the resulting suspension is low but is greatly increased by the addition of pyridoxal; secondly, a cell-free preparation can be made from normal cells left to autolyse in the presence of toluene, and if this preparation is left to age, its activity decreases but can be restored by the addition of pyridoxal phosphate.

The function of pyridoxal in amino-group transfer was first suggested by Snell,⁴ who showed that pyridoxal will react with

¹ Kritzmann & Kritzmann, 1937.

² Lichstein, Gonsky & Umbreit, 1945.

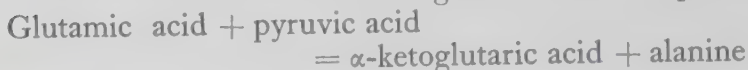
³ Lichstein & Cohen, 1944.

⁴ Schlenk & Snell, 1945.

glutamic acid to give pyridoxamine and α -ketoglutaric acid, and that the pyridoxamine can in turn react with a keto-acid to form the corresponding amino-acid and liberate pyridoxal again. These results were realised in a biological system by Bellamy *et al.*,¹ who showed that, although pyridoxamine cannot act as codecarboxylase, it is converted to a substance active as codecarboxylase if it is incubated with pyruvic acid and *Str. faecalis*. The reaction occurring is presumably:



Transaminase reactions have been investigated in detail in many cells,² but cell-free systems have been obtained which can catalyse only two reactions:



and there is consequently considerable doubt whether transamination can be held responsible for general amino-acid synthesis as originally suggested.

Amino-acid assimilation and metabolism by gram-positive bacteria

Many strains of pathogenic cocci have been found to be very deficient in the power to deaminate amino-acids,³ and the greater number of gram-positive organisms require a large number of amino-acids supplied as such in the medium. It has been shown that gram-positive bacteria are able to assimilate certain amino-acids from the medium and to concentrate these within the cell. Two cases⁴ have been studied in detail, viz. the passage of lysine and of glutamic acid across the cell wall of *Str. faecalis*; the former seems to occur by simple diffusion though a considerable concentration within the cell takes place, this being greater when the external concentration is low than when it is high. Thus when the external concentration is 25 $\mu\text{l./ml.}$ the internal concentration is increased by a factor of 15–20, whilst when the former is 300 $\mu\text{l./ml.}$ the increase is 4–5 times. The passage of glutamic acid, on the other hand, requires energy which can be supplied by glycolysis. In this case the ratio of the concentration of glutamic acid inside the cell to that in the external medium is about 50 when the external concentration is 10 $\mu\text{l./ml.}$ and falls to 3–4 when the external concentration is 300 $\mu\text{l./ml.}$ The passage of the two amino-acids can be further differentiated since the rate of entry of lysine

¹ Bellamy, Umbreit & Gunsalus, 1945.

² Herbst & Rittenburg, 1943.

³ Hills, 1940.

⁴ Gale, 1947.

is proportional to its external concentration whilst that of glutamic acid is constant for external concentrations above a certain low value, the rate-concentration relation being similar to that obtained for the rate-substrate concentration relation of an enzyme system. In either case the final concentration of the amino-acid achieved within the cell is markedly greater than that in the external medium with which it is in equilibrium. In the case of lysine, the internal concentration seems to depend also on the charge within the cell so that any process which alters this charge alters the amount of lysine bound by the cell.

In the case of glutamic acid the level of the free amino-acid measured within the cell is determined by the balance between the rate at which it is assimilated from the external medium and the rate at which it is metabolised.¹ Investigation of the action of various inhibitors has shown (1) that penicillin impairs the assimilation process without affecting the internal metabolism;² (2) that glutamic acid undergoes a metabolic change within the resting-cell and that this metabolism is inhibited by dyes of the triphenylmethane series;³ and (3) that this process can be impaired by the sulphonamide drugs.⁴ The precise nature of the metabolism which is inhibited by the triphenylmethane dyes is not yet certain though an intracellular enzyme system has been found which, in the presence of A.T.P., phosphorylates glutamic acid and is sensitive to crystal violet in concentration of the order of $M/4000$.⁵

The assimilatory processes can be investigated separately by the use of these inhibitors and it has been shown that the rate of assimilation of glutamic acid is approximately constant throughout the growth period though it falls rapidly after the cessation of growth.⁶ This is in agreement with the view which links protein synthesis with nucleic acid within the cell (see p. 148).

These processes have all been elucidated with gram-positive cocci and nothing is yet known of their application to gram-negative organisms or to those able to synthesise their amino-acids rather than depending on assimilation. It seems probable that the capacity of the gram-positive organism to concentrate amino-acids in the free state within the cell is a form of compensation for loss of synthetic ability. In this connection it is interesting that when *Staph. aureus* is trained to resist very high concentrations of penicillin (6000 units/ml.) it becomes gram-negative and loses the power to assimilate glutamic acid and acquires the power to synthesise all its amino-acid requirements.^{7, 8}

¹ Gale & Mitchell, 1947.

² Gale & Taylor, 1947 (1), (2).

³ Gale & Mitchell, 1947.

⁴ Gale, 1947.

⁵ Elliott & Gale, 1948.

⁶ Gale, 1947.

⁷ Bellamy & Klumck, 1947.

⁸ Gale & Rodwell, 1948.

CHAPTER VI

THE METABOLISM OF NUCLEIC ACID AND ITS DERIVATIVES

NUCLEIC acid forms the principal constituent of the nuclei of cells ; it also forms compounds of a salt-like character with proteins—in particular those such as the protamines and histones containing a predominance of basic groups. These are known as nucleoproteins and play a large part in actively multiplying cells ; they have also been shown to form the main constituent of both plant and animal viruses.

The existence of nuclei in bacteria was for long a matter of doubt,¹ though several earlier investigators claimed to have shown them.² Recently, however, Robinow, using the Giemsa stain, has clearly demonstrated nuclei in a number of common bacteria ; no mitosis has, however, been shown.³

Though the existence of nuclei in bacteria remained long a matter of doubt, bacterial cells contain a higher percentage of nucleic acid than any animal tissue except thymus, a characteristic shared with yeasts and moulds.^{4, 5}

TABLE 1

Organism	Percentage dry wt.		Purine N × 100
	Protein N	Purine N	Protein N
<i>Ster. nigra</i> . . .	4·41	0·41	9·2
<i>Asp. oryzae</i> . . .	3·94	0·31	7·8
<i>Bac. de la fléole</i> . . .	5·71	0·43	7·5
Lactic bacillus . . .	9·92	1·15	11·5
Tubercle	4·46	0·32	7·1
Yeast ("Fala") . . .	6·69	0·77	11·5
Yeast (Baker's) . . .	5·97	0·66	11·0
(For comparison)			
Muscle	—	—	2·3
Liver	—	—	4·5

Nucleic acid occurs *in vivo* in loose chemical combination with basic proteins, the complex being known as nucleoprotein. The general method of preparation is to subject the defatted cell either

¹ Lewis, 1941.

² Dobell, 1911.

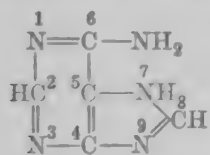
³ Robinow, 1945.

⁴ Terroine & Szucs, 1930 (1), (2).

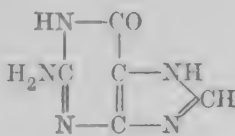
⁵ Le Breton & Kayser, 1926.

before or after mechanical disruption to mild alkaline hydrolysis, which breaks the nucleic acid-protein combination, and then to precipitate the proteins by bringing them to their respective isoelectric points or by precipitants. The nucleic acid is obtained by precipitation at about pH 4, usually in the presence of ethanol.

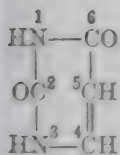
Yeast nucleic acid after gentle hydrolysis gives rise to the following components: two purine bases adenine and guanine, two pyrimidine bases cytosine and uracil, the pentose sugar ribose in the furanose form, and phosphoric acid. Thymus nucleic acid differs from that of yeast in containing thymine in place of uracil and 2-deoxy-*d*-ribose in place of *d*-ribose.



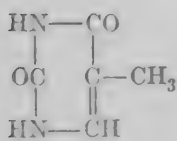
Adenine



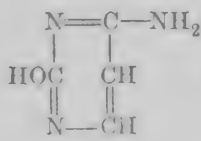
Guanine



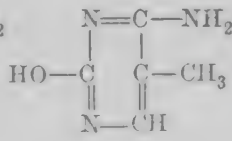
Uracil



Thymine



Cytosine



Methyl Cytosine

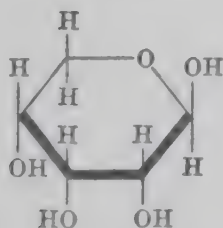
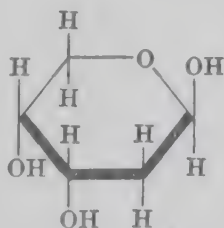
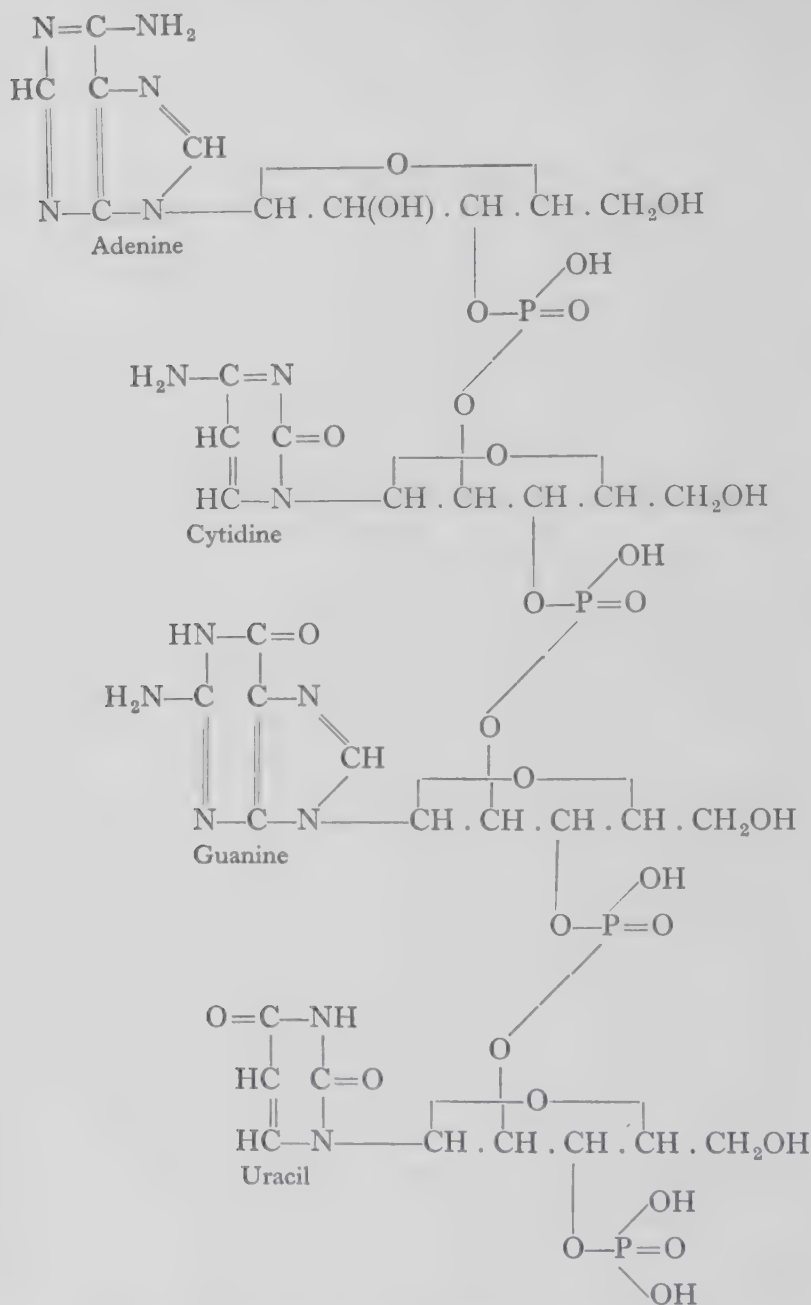
 β -*d*-ribose β -2-deoxy *d*-ribose

FIG. 1

The property of staining with Feulgen's reagent, that is of restoring the colour of a fuchsine solution decolorised by sulphurous acid,¹ is generally attributed to nucleic acid containing deoxyribose, though the presence of that substance has not always been correlated with a positive Feulgen reaction.

When yeast nucleic acid is subjected to gentle hydrolysis four nucleotides are obtained in equimolecular proportions, each containing one of the four bases characteristic of this nucleic acid.

¹ Feulgen & Rosenbeck, 1914.

FIG. 2¹—Tetranucleotide of yeast nucleic acid (formula of Levene)¹ Greenstein, 1944.

This has led to the acceptance of the tetranucleotide structure of nucleic acid put forward by Levene, in which the phosphate is linked to the carbon of the ribose in position 3 whilst the carbon 1 of ribose is attached to the base in position 3 in the case of the pyrimidines, and in position 9 in the case of the purines. Levene's formula is shown in Fig. 2.¹ The molecule of nucleic acid consists of a tetranucleotide units united through ester linkages.

Bacterial nucleic acids

Bacterial nucleic acids have been studied by (1) U.V. photography, (2) staining after different treatments, (3) chemical analysis.

Method 1 depends on the fact that purines and pyrimidines absorb U.V. light very strongly, absorption being at a maximum at 2600 Å; this property is used, not only as a means of demonstrating the presence of nucleotides, but also as a method for estimating them. A full account of this technique as applied to bacteria is given by Malmgren and Hedén.²

The second method is by the use of the Giemsa stain and has been developed by Robinow.³ This method is not adapted to quantitative work but has the advantage of distinguishing between the two types of nucleic acid. When bacterial cells without pretreatment are stained by this method they show a diffuse dark stain throughout; if, previous to staining, they are washed with *N* HCl in the cold for 5 to 7 minutes the whole of the ribose nucleic acid and about 7.5% of the desoxy-type is washed out;⁴ the cells then show darkly stained nuclei in a clear cytoplasm. Thus the ribose nucleic acid is seen to be located in the cytoplasm and the desoxy type in the nucleus. This method gives useful rough comparisons of the relative amounts of the two acids present in different phases of the growth cycle and in different nutritive conditions.

The chemical method of estimating the two nucleic acids consists in a pretreatment of the bacterial suspension for the removal of fats and lipoids followed by the extraction of the soluble nitrogenous compounds—nucleotides, nucleosides, purines and pyrimidines—by the use of 5% trichloroacetic acid in the cold. The cells thus treated contain the two nucleic acids which can then be extracted by 5% trichloroacetic acid at 90°. The total nucleic acid in this extract can be estimated either by precipitating the purine bases by copper⁵ or by estimation of organic phosphorus.⁶ The two nucleic acids can be separated by treating the cells, after the

¹ Levene & Barr, 1931.

² Robinow, 1945.

³ Vendrely & Sarciron, 1944.

⁴ Malmgren & Hedén, 1947 (1).

⁵ Vendrely & Lipardy, 1946.

⁶ Schmidt & Tannhauser, 1945.

removal of fat and soluble nitrogenous compounds, with *N* alkali which dissolves both types; the desoxynucleic acid can then be reprecipitated by making *N* with respect to HCl leaving the ribose type, or its decomposition products, in solution.¹ Alternatively use can be made of the specific nucleases^{2, 3} for the two types of nucleic acid, both of which are prepared from ox pancreas. Results obtained by these different methods corroborate each other satisfactorily.

In bacteria the ribose nucleic acid is quantitatively greater than the desoxy type; the former is distributed through the cytoplasm whilst the latter is confined to the nucleus where it is combined with basic proteins. The view due to Caspersson⁴ and his school, and widely accepted, is that the synthesis of all protein in the cell occurs in association with nucleic acid and is conditioned by it; the protein of the nucleus is formed in association with the desoxy-ribose nucleic acid and forms part of the genes and hence of the hereditary mechanism of the cell; the protein of the cytoplasm is associated with ribose nucleic acid. Table 2 gives the amounts of both sorts of nucleic acid found in some bacterial cells.

TABLE 2⁵
NUCLEIC ACID CONTENT OF SOME MICRO-ORGANISMS (% DRY WT.)

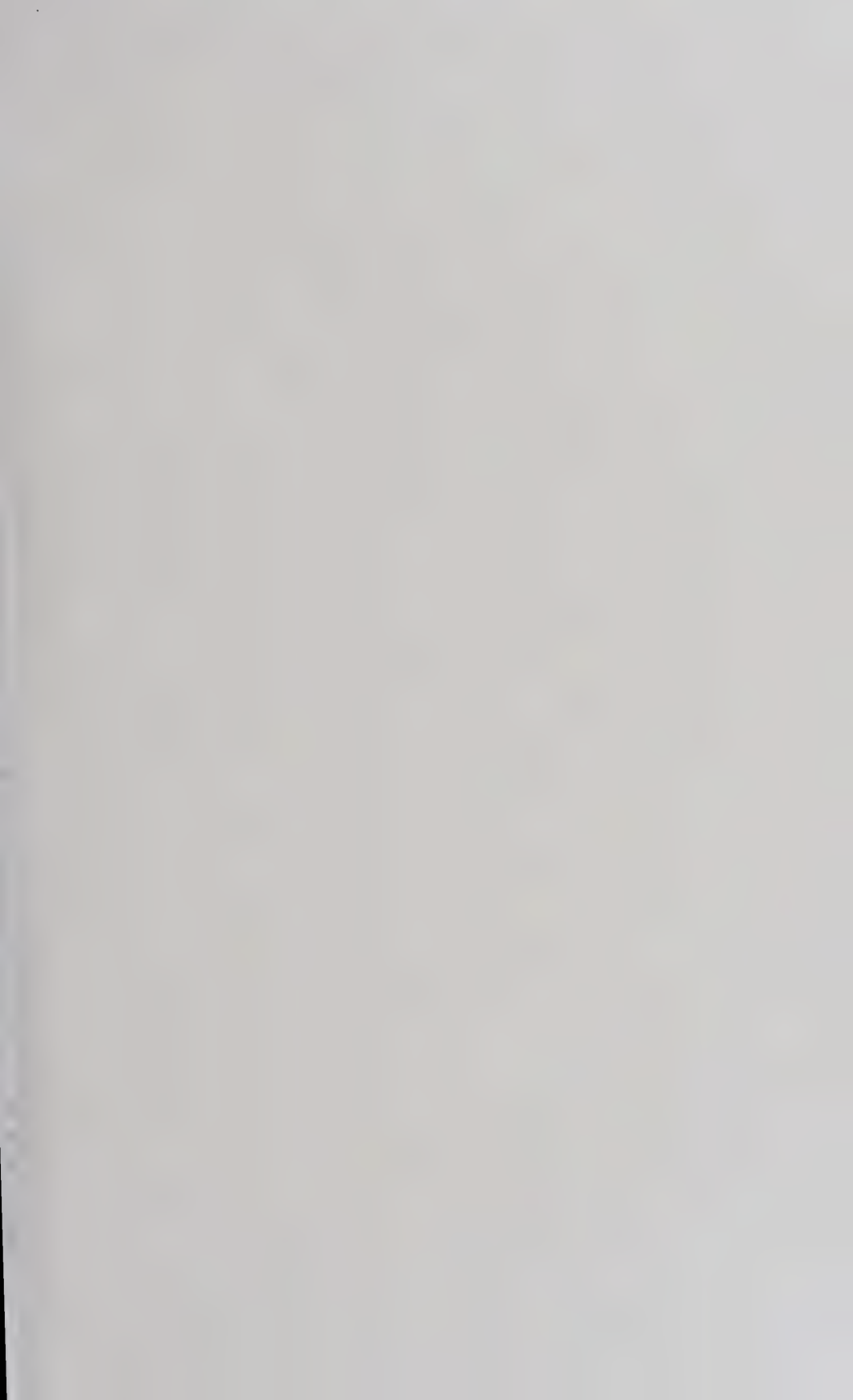
Organisms	Total N	Total Protein	Nucleic Acid			
			Total	D.R.N.A.	R.N.A.	D.R.N.A./R.N.A.
<i>Staphylococcus</i> .	13.95	75.50	11.57	2.82	8.75	0.24
<i>Bac. anthracis</i> .	10.00	58.00	4.35	1.15	3.20	0.26
<i>Bact. typhosum</i> .	14.61	78.50	12.84	3.72	9.12	0.29
Coliform (smooth). .	14.20	74.90	13.90	4.17	9.73	0.30
Coliform (smooth). .	14.40	76.80	13.12	4.40	8.72	0.34
Coliform (rough). .	14.78	77.60	14.67	4.24	10.43	0.29
Coliform (smooth). .	13.60	72.50	12.37	3.78	8.59	0.30
Coliform (rough). .	13.61	71.00	13.98	3.89	10.09	0.28
<i>Aertrycke</i> (smooth) .	14.48	82.05	8.40	3.00	5.40	0.36
Yeast (baker's) .	6.50	36.30	4.26	0.31	3.95	0.07
Yeast (champagne) .	5.90	33.10	3.73	0.52	3.21	0.14

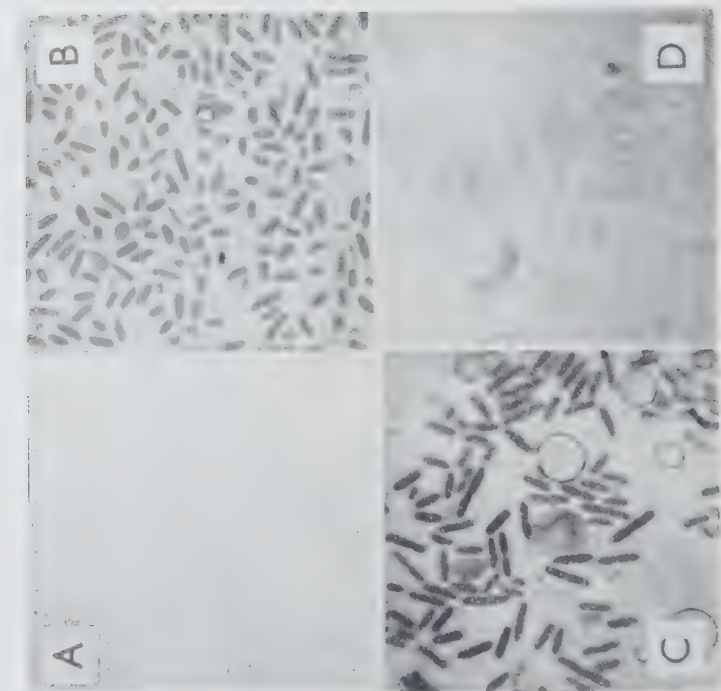
Nucleic acid and the growth cycle

By the quantitative method of U.V. microphotography⁶ it has been shown that the total amount of nucleotide material in the bacterial cell is a function of the growth rate, i.e. of the rate of protein synthesis. Cells removed from an 18- to 20-hour culture show very little absorptive material when photographed at 2570 Å. Before cell division starts nuclear material accumulates in the cell

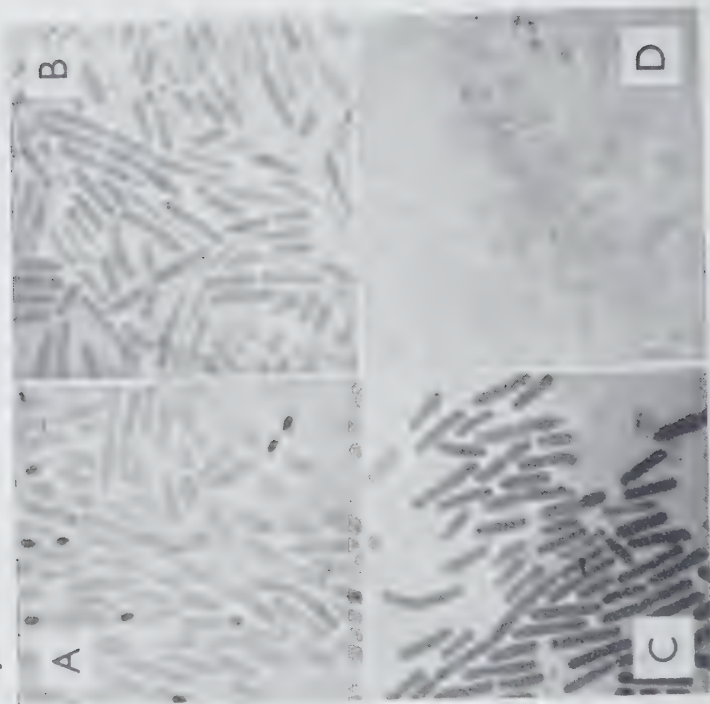
¹ Schmidt & Tannhauser, 1945. ² Kunitz, 1939. ³ McCarty, 1946.

⁴ Caspersson, 1947. ⁵ Vendrely, 1946. ⁶ Malmgren & Hedén, 1947 (1).





I

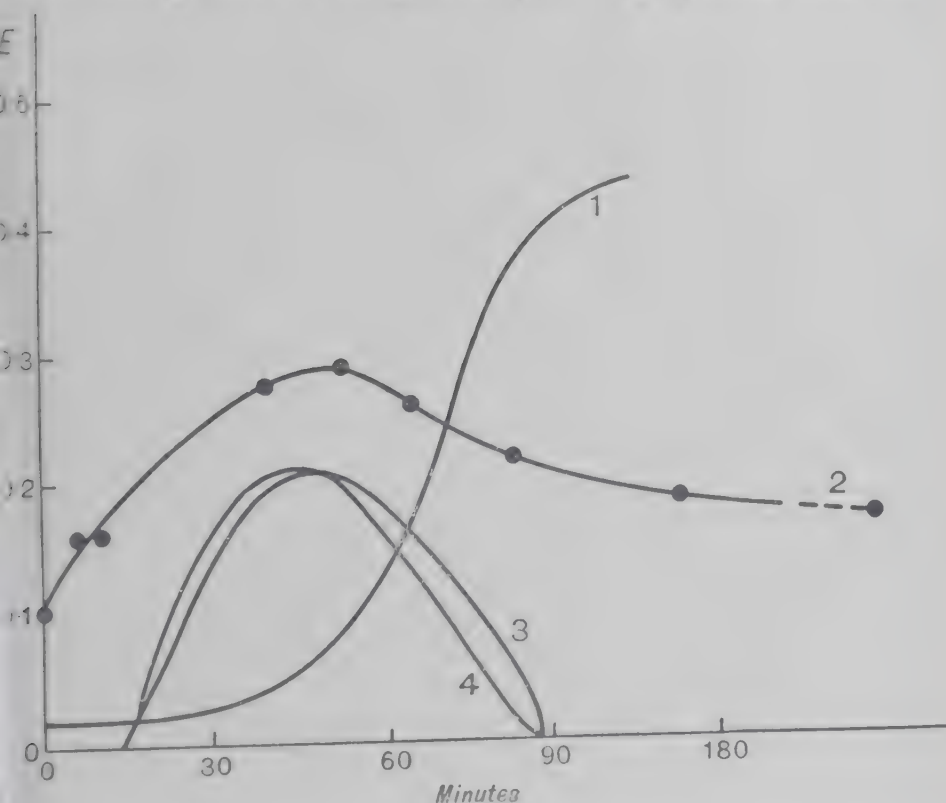


II

Microphotographs of I *E. coli* and II *B. cereus* at 2570 Å.

A=18-hour agar culture, B=the middle of the lag phase, C=the beginning of the logarithmic phase, D=phase of decline.

and by the middle of the lag phase the cells are moderately absorptive; by the beginning of the logarithmic phase the amount of nuclear material is at its height and thereafter declines (see Figs. 3 and 4).¹ Fig. 3 shows the large differences in nucleotide material at different periods of the growth cycle. Fig. 4 shows how this

FIG. 4¹

is related to multiplication rate. Curve 1 gives the number of bacteria present plotted against time. Curve 2 gives the extinction due to individual bacteria, each point corresponding to the mean value obtained from 200 cells. Curve 3 ($\frac{dn}{dt}$, where n is the number of bacteria and t the time) represents the rate of multiplication and is thus a measure of protein synthesis. Curve 4 is obtained from curve 2 by subtracting the amount of absorptive material originally present and therefore represents the increase in nuclear material occurring during the growth period.

These results show that, after sowing resting cells into fresh medium, a period of latency occurs, during which no cell division takes place (see p. 159). This time is occupied in accumulating nuclear material within the cell; when this has reached a certain

¹ Malmgren & Hedén, 1947 (1).

limit, cell division starts and both nuclear material and the rate of cell division increase proportionately till the logarithmic phase is past, when both decline, so that by the end of the growth cycle the amount of nuclear material has returned to the level from which it started. Thus the rate of cell division and hence of protein synthesis is closely proportional to the amount of nuclear material present. These results were obtained with gram-negative cells but similar results were found with gram-positive varieties.¹

No bacterial nucleic acids have been studied in the same detail as those of yeast and thymus but information regarding some of them is now available.

The nucleic acid of the tubercle bacillus has been examined both in the cells and in the culture fluid. In the former case 8.4 g. nucleic acid, known as tuberculinic acid, was isolated from 2 kilos of dried material; sugar was demonstrated but not characterised. Adenine, guanine, cytosine, and thymine were isolated with small amounts of the unusual pyrimidine, methyl cytosine.^{2, 3}

Nucleic acid has also been isolated from the culture fluid in which the tubercle bacillus has been grown for the production of tuberculin. The bases of this nucleic acid were not characterised but the sugar was identified as desoxyribose. It is not clear whether this nucleic acid is identical with that of the cell nucleus previously described.⁴

The nucleic acid of the allied non-pathogen *M. phlei* (the Timothy Grass Bacillus) has also been studied;⁵ adenine, guanine, uracil and cytosine were identified and a pentose—not desoxyribose—was demonstrated. This nucleic acid therefore resembles the yeast type whilst that of the closely related *M. tuberculosis* resembles the thymus type. It may be pointed out, however, that the nucleic acid of only one species of yeast has so far been studied and it may well be that the ribose type is not characteristic of this group as a whole; recent work also shows that most organisms contain both types.⁶ The nucleic acid from the typhoid bacillus of mice⁷ contains guanine and adenine in equimolecular proportions, also thymine, cytosine and uracil; qualitative tests for ribose and for desoxyribose (Feulgen) were obtained from which it was concluded that the nucleic acid of this organism is of a mixed type.⁸ A similar observation has been recorded for *C. diphtheriae*.⁹

Nucleic acid and the gram stain

The gram stain was introduced by Christian Gram¹⁰ in 1884

¹ Malmgren & Hedén, 1947 (4).

² Johnson & Coghill, 1925.

⁶ Henry *et al.*, 1945.

⁹ Coghill & Barnes, 1932.

² Brown & Johnson, 1923.

⁴ Seibert, 1944.

⁷ Akasi, 1938.

¹⁰ Gram, 1884.

⁵ Coghill, 1931.

⁸ Ibid.

and has since been regularly used as a differentiating test for bacteria. Gram-positive organisms after heating fix crystal violet after subsequent treatment with aqueous iodine, and the dye remains fixed after moderate washing with ethanol. Gram-negative organisms do not fix the dye in these conditions and it is washed out with ethanol and the organisms can then be counterstained.

The character of the bacterial substance responsible for fixing the dye has recently been elucidated. It was shown that pneumococci killed by maintaining at pH 4.2 overnight and subsequently incubating at neutrality undergo some autolysis and discharge nucleic acid and a nucleoprotein into the medium; the nucleic acid obtained from the medium was decomposed by pancreatic ribonuclease and was therefore believed to be of the ribose type. The organisms after this treatment were gram-negative; they gave a positive Feulgen reaction but no desoxyribonucleic acid was found.¹

Very important light has been thrown on the chemical structure of gram-positive organisms by Henry and Stacey.² These workers showed that if aqueous suspensions of gram-positive organisms were treated with 2% sodium cholate in the presence of oxygen (or by ribonuclease) they lost their power to fix the dye complex and became gram-negative. From the aqueous extract the magnesium salt of ribonucleic acid was prepared and it was shown that it is to this substance that the gram staining is due. Provided the stripped cells were kept reduced by 1% formalin the magnesium ribonucleate could be replated on to the cytoskeletons, which again became gram-positive.³ The magnesium salt of desoxyribonucleic acid was ineffective but ribonucleates from one species could be replated on to another; attempts to plate cells originally gram-negative were always unsuccessful.

In the case of yeast the stripping process first removed an outer layer of stainable material, disclosing a stippled surface and a gram-positive nucleus; on further treatment the latter also was stripped. Both nucleus and surface could be replated.

The gram-positive organism *S. salicarius*, when grown in minimal amounts of magnesium or on acid media, became gram-negative and on subculture gave gram-negative decapsulated cells. This observation should be compared with that of Avery *et al.*⁴

The gram-negative cytoskeletons gave a strong Sakaguchi reaction (test for arginine), indicating the presence of basic proteins; this suggested that the ribonucleic acid might be combined as a nucleoprotein in the cell. This was shown to be the case by auto-

¹ Thompson & Dubos, 1938.

² Henry & Stacey, 1943.

³ These observations later confirmed Bartholomew & Umbreit, 1944.

⁴ Avery *et al.*, 1944.

⁵ Sakaguchi, 1925.

lysing gram-positive cells (*Cl. welchii* and yeast) at pH 8.0 and 37°, when a nucleoprotein giving a gram-positive reaction was separated. On splitting this into protein and nucleic acid neither component fixed the stain, but on recombining the two with Mg in the presence of formaldehyde a gram-positive complex was reformed. It was again shown that this procedure was not possible with gram-negative organisms.¹ It was noted that the ratio of the two types of nucleic acid differed in the gram-positive and gram-negative organisms examined, the former giving a ribose/desoxyribose ratio of more than 8/1, the latter 1.3/1.

The decomposition products of nucleic acid

Besides the nucleic acids proper (polynucleotides), various decomposition products occur in the cell. These are (1) mononucleotides, compounds containing one molecule each of base, pentose and phosphoric acid; (2) nucleosides, compounds of base and pentose; and (3) free bases.

When nucleic acid is added to bacterial suspensions it is decomposed into its components. MacFadyen² showed that a 3% solution of yeast nucleic acid added to a suspension of *Bac. subtilis* was disrupted to the extent of 80% in 48 hours at pH 6.8. The preparation was fractionated into undecomposed nucleic acid by the use of uranyl chloride 1.25% in 10% trichloroacetic acid; at pH 6.8 the nucleic acid was precipitated whilst the nucleotides and smaller units were left in solution; the supernatant solution was then precipitated with lead acetate at pH 6.8, which removed nucleotides. From this procedure four fractions were obtained: (1) the sodium carbonate solution of the uranyl trichloroacetic precipitate; (2) the filtrate from this; (3) the supernatant after treatment with neutral lead acetate and removal of lead; (4) the unfractionated culture. Each fraction was analysed for total N inorganic and total P; the results are summarised in Table 3. Comparison of columns (f) and (g) shows that the nucleotides were decomposed mainly to phosphoric acid ester of ribose and bases. The ratio of total N in fraction (3) to that in fraction (2) (column (e)) gives the proportion of nucleotides broken down after separation from nucleic acid. The decomposition of nucleotides can occur in three ways: if P represents phosphate, R ribose and N base,

1. $PRN \rightarrow P + RN$
2. $PRN \rightarrow P + R + N$
3. $PRN \rightarrow PR + N$

The ratio of inorganic phosphate to total phosphate in fraction

¹ Henry *et al.*, 1945.

² Macfadyen, 1934.

TABLE 3¹EXTENT AND QUALITY OF DISINTEGRATION OF NUCLEOTIDES BY *Bacillus subtilis*

pH	Total N		Nucleotide hydrolysis (c)	Total P	Inorganic P	Nucleotide hydrolysis	
	Fraction 2 (a)	Fraction 3 (b)		Fraction 2 (d)	Fraction 2 (e)	(f)	(g)
	mg.	mg.	per cent	mg.	mg.	per cent	per cent
6.0	14.9	11.5	77	8.72	2.67	31	46
6.6	22.9	18.3	80	13.52	3.54	26	54
7.2	20.8	16.6	80	12.46	3.39	27	53
7.8	14.7	7.4	50	8.51	0.06	1	49
8.2	9.4	2.8	30	5.43	0.00	0	30

Nucleic acid, 3.2% ; pH as shown ; time of incubation, 48 hours.

$$c = (b/a) \times 100 ; f = (e/d) \times 100 ; g = c - f.$$

c represents nucleotide hydrolysis of all three types ; f, nucleotide hydrolysis of both types yielding inorganic phosphorus ; g, nucleotide hydrolysis of the type yielding phosphoric acid ester of ribose and free nitrogenous base.

(2) gives the proportion of nucleotide broken down by methods 1 and 2 ; this is given in column (f). The nucleotide broken down by 3 is given by the difference between percentage hydrolysis given by N column (c) and that by P column (g). It is seen that at acid and neutral values of pH a considerable amount of decomposition of ribose phosphate occurs which is absent at alkaline reaction. This may be in part due to the spontaneous decomposition of ribose phosphate at acid reactions apart from bacterial action.

The nucleotide fractions of bacteria

Besides nucleic acid itself various nucleotides, nucleosides and free bases are present in the bacterial cell ; a study of these is due to Boivin and Mesrobian.² The method employed was :

1. Treatment of washed bacteria centrifuged to form a thick paste with an equal volume of N/2 trichloroacetic acid at about 0° for 3 hours ; the whole is then centrifuged. The supernatant contains ammonium and mineral salts, sugars, amino-acids and purine derivatives (mononucleotides, nucleosides and free bases), but no nucleic acid. The free purines are liberated from both the supernatant and the precipitate by 8 hours' hydrolysis in N HCl at 100° and separated by precipitation with copper.³ The total purines (1) and acid-soluble purines (2) are thus obtained ; the purines of the nucleic acid are obtained by difference (1-2).

¹ Macfadyen, *J. Biol. Chem.* (1934), 107, 306.

² Mesrobian, 1936.

³ Le Breton & Kayser, 1926.

The acid-soluble fraction A is separated into (1) mononucleotides, (2) nucleosides, and (3) purine bases. (1) is precipitated by uranyl acetate and after washing, etc., hydrolysed by acid; the solution then contains free purines originally present as nucleotides, which (after removal of uranium) are precipitated by copper and estimated by total N. Fraction (3) (purine bases) is obtained by precipitation with copper without previous treatment. The total purines of the acid-soluble fraction are obtained by copper precipitation following acid hydrolysis; the nucleosides are obtained by difference ($A - (1 + 3)$). The nucleosides can be estimated directly by precipitation with lead due to the fact that lead acetate at pH 5.5 precipitates mononucleotides which can be removed; the filtrate with excess of lead is then made alkaline, thus precipitating the nucleosides.

As pointed out by Boivin,² the procedure outlined above fails to distinguish between the N of the purine nucleus and that of the amino-purines adenine and guanine; the latter are estimated by the application of van Slyke's method for amino-N.

From the free purine bases of the acid-soluble fraction methyl purines and uric acid were absent; guanine, adenine, xanthine and hypoxanthine were separated and estimated; for details of the procedure the original papers must be consulted.² Tables 4 and 5 give the results obtained. Table 4 shows that with fresh bacteria the acid-soluble N forms an important fraction of the total, i.e. 10–20%. The ammonia-N and amino-N together make up about half the acid-soluble N. The total purine-N forms from 0.20 to 0.30% of the fresh bacteria; this is higher than in

TABLE 4³

Bacteria	Purine-N (total) per 100 parts bacteria (wet wt.)	Dry wt. bacteria per cent	Total N percent dry wt.	Total purine-N as percent of total N	Acid-soluble N as percent of total N	Acid-soluble purine-N as percent of total acid-soluble N	NH ₃ -N as percent of acid-soluble N	Amino-N as percent of acid-soluble N
<i>Staph. aureus</i> .	0.29	24.6	12.1	9.4	17.0	3.9	18.3	32.5
<i>B. subtilis</i> .	0.18	20.8	11.2	7.8	14.7	9.1	15.0	26.9
<i>Proteus vulgaris</i>	0.25	19.9	13.9	9.1	9.1	23.9	29.7	19.8
<i>Micrococcus prodigiosus</i> .	0.29	26.0	12.6	9.1	9.8	10.8	19.7	30.1
<i>B. pyocyaneus</i> .	0.23	21.2	12.7	8.7	8.3	10.6	31.4	35.4
<i>B. coli</i> .	0.27	22.5	13.7	8.7	4.7	11.6	29.6	27.2

¹ Boivin, 1929, *Thesis*.² Mesrobianu, 1936, *Contribution a l'étude des corps puriques de la cellule bactérienne*, p. 138.³ Ibid.

TABLE 5

Bacteria	Acid-soluble purine-N in 100 parts of total purine-N	In 100 parts of acid-soluble purine-N		
		N of free purines	N of nucleo- sides	N of nucleo- tides
<i>Staph. aureus</i> .	7.2	5.7	58.2	36.1
<i>B. subtilis</i> .	19.0	2.0	86.1	11.7
<i>Proteus vulgaris</i> .	27.0	3.0	82.1	14.9
<i>Mic. prodigiosus</i> .	10.5	6.1	58.2	35.7
<i>B. pyocyaneus</i> .	10.3	3.7	55.3	41.0
<i>B. coli</i> .	6.2	5.1	59.1	35.8

the case of any animal tissue except thymus, which reaches 0.45% wet weight. The total purine-N is about 10% of the total bacterial N; this is also higher than in any animal tissue except thymus; see Table 6.

TABLE 6^{1, 2}

Tissue	Purine-N in 100 parts of	
	Fresh tissue	Total N
Thymus . .	0.45	14.1
Spleen . .	0.16	5.0
Liver . .	0.12; 0.145	3.7; 4.5
Muscle . .	0.06; 0.075	1.9; 2.3

Table 5 shows that 75-95% of the Purine-N is present in the acid-insoluble form, i.e. as polynucleotides; of the acid-soluble fraction the nucleosides are quantitatively the most important and the free purines the least.

Distribution of purines

The distribution of the purines was studied as follows. The richloroacetic extract from 200 g. (wet wt.) of bacteria was separated into nucleotides and nucleosides and hydrolysed. The solution of purines thus obtained contained no methylated purines and traces only of uric acid; four purine bases were separated and identified and their approximate amounts estimated. Guanine was obtained by precipitation with ammonia; adenine in the residue as picrate; hypoxanthine and xanthine together in the residue by copper precipitation after removal of the picric acid.

As is seen from Table 7, adenine predominates in both nucleotides and nucleosides, but the other bases are represented. The purine bases present in the nucleic acid of yeast are adenine and guanine in equimolecular proportions; supposing the nucleic

¹ Barian & Schur, 1960.² Terroine & Ritter, 1927.

TABLE 7¹
 DISTRIBUTION OF PURINE BASES

Organism	Base	Mols. per cent as	
		Nucleotides	Nucleosides
<i>Bact. coli</i> .	Amino-purines { guanine adenine	6.5 } 91.0 84.5 }	5.3 } 74.9 69.6 }
	Oxypurines : hypoxanthine + xanthine	9.0	25.1
<i>Proteus</i> .	Amino-purines { guanine adenine	10.7 } 86.6 75.9 }	9.4 } 72.3 62.9 }
	Oxypurines { xanthine hypoxanthine	— 13.4	5.0 } 27.7 22.7 }
<i>Bact. aertrycke (Rough)</i>	Amino-purines { guanine adenine	0.0 } 57.7 57.7 }	0.0 } 51.7 51.7 }
	Oxypurines { xanthine hypoxanthine	36.4 } 42.3 5.9 }	42.2 } 48.3 6.4 }

acid of bacteria to have a similar structure the predominance of adenine in the acid-soluble fractions suggests that it may have some origin apart from the enzymic decomposition of nucleic acid. The exact structure of the nucleotides of bacteria is uncertain ; those of which the structure is already known are muscle adenylic acid (p. 69) and yeast adenylic acid ; the former (adenosine-5-phosphoric acid) is present in the acid-soluble fraction of yeast cells and of muscle, the latter (adenosine-3-phosphoric acid) is obtained by the hydrolysis of yeast nucleic acid. In yeast adenosine-5-phosphoric acid can be decomposed autolytically, giving adenosine which can be phosphorylated by the yeast enzymes, giving (1) adenosine-5-phosphoric acid and adenosine di- and triphosphate.² In addition there is present in yeast and in muscle adenylyl pyrophosphate (A.T.P.) (p. 69) whose part as a phosphate carrier in fermentation has already been discussed. A.T.P. has been obtained from the water-soluble fraction of *Staphylococcus*, *V. Metschnikovi* and *Asotobacter*³ and from the trichloroacetic extract of various bacteria by precipitation with barium.⁴ Lutwak-Mann definitely showed the presence of A.T.P. in *Bact. coli* as follows : the trichloroacetic extract was precipitated with barium, the precipitate dissolved in hydrochloric acid, freed from barium and ammonia and the pyrophosphate-P estimated by Lohmann's method and the adenine amino-N by the specific deaminase obtained from frog muscle ; adenylic acid was absent from this preparation.⁵

¹ Mesrobian, 1936.³ Lohmann, 1928 (1), (2).⁵ Lutwak-Mann, 1936.² Ostern *et al.*, 1937 and 1938 (1), (2).⁴ Mesrobian, 1936.

The enzymic decomposition of purine compounds

The interchanges occurring in the purine compounds during the autolysis of bacteria have been studied by Boivin and Mesrobian.^{1, 2} A thick bacterial suspension (200 mg. wet bacteria per ml.) buffered at pH 7.0 was divided into two portions. The first was treated in the usual way with trichloroacetic acid and the purine-N of the precipitate and supernatant estimated as usual. The second part was autolysed at 37° in the presence of toluene for one week and then treated in the same way; the results are seen in Table 8. The small loss in total purine-N is attributable

TABLE 8³

Fraction of purine-N estimated	Purine-N of different fractions in 1000 parts of total purine-N of fresh bacteria		
	Before autolysis	After autolysis	Difference
<i>Bact. coli</i>			
Free purines . . .	3.3	41.2	+ 37.9
Nucleosides . . .	30.4	94.2	+ 63.8
Nucleotides . . .	23.6	47.0	+ 23.4
Nucleic acid . . .	942.7	773.4	- 169.3
	1000.0	955.8	- 44.2
<i>Proteus</i>			
Free purines . . .	5.9	67.3	+ 61.4
Nucleosides . . .	185.9	312.7	+ 126.8
Nucleotides . . .	42.8	93.0	- 50.2
Nucleic acid . . .	765.4	474.7	- 290.7
	1000.0	947.7	52.3
<i>Staphylococcus</i>			
Free purines . . .	4.2	74.6	+ 70.4
Nucleosides . . .	38.6	84.5	+ 45.9
Nucleotides . . .	28.0	38.2	+ 10.2
Nucleic acid . . .	929.2	781.5	- 147.7
	1000.0	978.8	- 21.1

to the deamination of the amino-purines. Apart from this the nucleic acid diminishes and the acid-soluble purine fractions increase during autolysis; also the amino-purines of each of the acid-soluble fractions diminish, whilst the oxypurines increase. For this purpose, the ammonia and amide-N having been estimated, the acid-soluble fraction was separated into the nucleotide fraction and the nucleoside + free purine fraction, in each of which the following were estimated:

(1) Total purine-N (A). (2) The amino-N of the amino-purines (B). Hence (3) the nuclear N of the amino-purines

¹ Mesrobian, 1936.² Boivin & Mesrobian, 1934.³ Mesrobian, 1936.

(4B). This gives the change in the ratio of amino-purines to total purines resulting from autolysis (Table 9).

TABLE 9¹

Organism	Fraction	Nuclear-N of amino-purines Nuclear-N of total purines	
		Before autolysis	After autolysis
<i>Bact. coli</i>	Nucleotides	88.6	61.7
	Nucleosides + free purines	60.5	29.1
	Total acid-soluble	77.5	38.3
<i>Proteus</i>	Nucleotides	92.5	52.4
	Nucleosides	75.0	45.1
	Total acid-soluble	78.0	46.7

The bacterial enzymic decomposition of nucleic acid derivatives added to the medium also occurs. Lutwak-Mann² has shown that the following compounds are deaminated by washed suspensions of *Bact. coli* and other organisms of the same group: (1) adenine; (2) adenosine; (3) adenylic acid (yeast and muscle); (4) guanylic acid; (5) adenylyl pyrophosphate. (3), (4) and (5) as well as inosinic acid are dephosphorylated; hypoxanthine was isolated as an end-product from adenosine, adenine, inosine and inosinic acid. *Ps. pyocyanea* produced only deamination but lacked dephosphorylating enzymes.

¹ Mesrobianu, 1936, *Thesis*, p. 166.

² Lutwak-Mann, 1936.

CHAPTER VII

GROWTH AND NUTRITION

PHASES OF BACTERIAL GROWTH IN CULTURE MEDIA

BACTERIAL growth is usually estimated by a "viable count." This consists in transferring a small volume from the culture to a known volume of sterile Ringer's solution or other liquid which is non-toxic and non-nutrient ; after successive dilutions in the same diluent, a known volume is finally transferred to sterile nutrient agar which is either poured on to a plate or rolled in a thin layer inside a test-tube (roll tube method) ; after incubation each viable cell originally present is represented by a colony ; these are counted and the number of viable organisms in the original culture calculated. In order to obtain a total count a drop, after suitable dilution, is mounted in a special chamber of known volume and counted under the microscope, either after staining or by the use of dark ground illumination ; full critical description of both these methods is given by Wilson.¹ A measure of growth may also be obtained by dry weight or by a determination of total nitrogen by micro-Kjeldahl method ; in the latter case it is necessary to be sure that the nitrogen content of the organism is constant. With organisms which do not clump the growth may be measured in a turbidimeter, preferably by means of a photoelectric cell ; the method must be checked against total count or dry weight for each organism used.

When culture medium is seeded with a small inoculum and the total count plotted against time, a curve of the form shown in fig. 1 is obtained.

Fig. 1 shows eight phases of growth :

1. An initial stationary phase during which no multiplication occurs.
2. The lag phase or period of positive growth acceleration during which the rate of multiplication increases with time.
3. The logarithmic growth phase during which the rate of increase remains constant.
4. The phase of negative growth acceleration during which the rate of multiplication decreases.

¹ Wilson, 1922.

5. The maximum stationary phase during which no change in population occurs.
6. The phase of accelerated death during which the numbers are falling off with increasing rapidity.
7. The logarithmic death phase during which the rate of death is constant.
8. The phase of decreasing death-rate.)

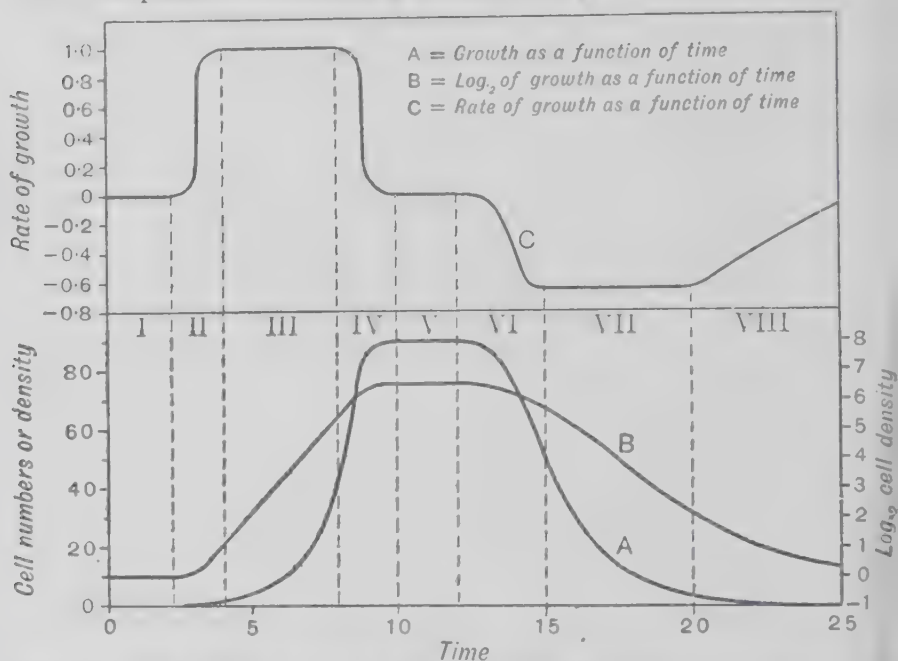


FIG. 1.—Typical growth curve of bacteria (Roman numerals and dotted vertical lines mark the growth phases)¹

The lag phase

(This comprises all that period (1 and 2) between the moment of inoculation and the establishment of a constant and maximum rate of cell division.) A large amount of work has been done on this phase in order to explain why the viable cell when removed to fresh media does not immediately begin to divide at the maximum rate. In the vast majority of cases growth has been estimated by cell numbers, either total or viable, and it has been assumed that growth, i.e. increase in cell material, is proportional to increase in cell numbers. Actually, during the logarithmic and later phases this is true, but in the lag phase this relation does not hold.) This was first discovered by Henrici, who showed that in the cases of *B. megatherium* and *Bact. coli* the average cell size increased very markedly during the lag phase, especially along the major axis.

¹ Monod (1942), *La Croissance des cultures bactériennes* (Hermann et Cie, Paris).

the average length of *B. megatherium* at the end of lag being six times that of the inoculated cells. This period of increased size is also marked by great fluctuations in form, as shown by the area-length index $\left(\frac{\text{area}}{\text{length}}\right)^1$. On passing into the logarithmic phase, the cells decrease in size, approximating to that of the inoculation; simultaneously, the great fluctuations in form cease. In some instances the largest cells occur in the logarithmic phase. Fig. 2, from Henrici's studies (on *Bact. coli*), illustrates these points.

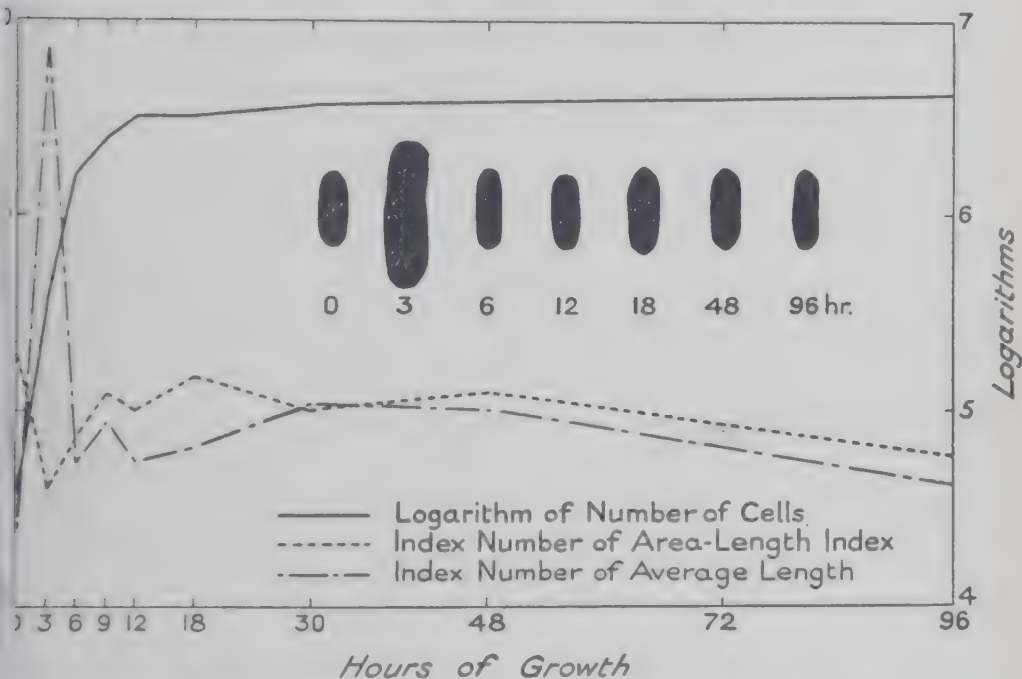


FIG. 2.—Graph illustrating variations in form and size of *Bact. coli* with the age of the culture grown on beef extract agar¹

These results suggest that some cause operates in fresh media favourable to increase of cell material but inhibitory to cell division, leading the majority of cells to attain an abnormal size before plitting occurs. Large inocula tend to decrease this effect, the average maximum size being smaller and attained earlier than with small inoculations.² Moreover, if in the latter case the culture is reinoculated into fresh medium before the critical point of maximum size is reached, the cells attain a larger size, and the critical point occurs later than in the case of the parent culture.³ Hence it appears that the size of the cell is partly conditioned by the density of the cell population. (Another controlling factor is con-

¹ Henrici, *Proc. Soc. exp. Biol. and Med.*, 1923, **21**, 216.

² *Ibid.*, 1921, 1923 (1), (2).

³ *Ibid.*, 1923 (2).

centration of nutrient material; in broth agar of one-quarter the normal strength, the maximum size of the organisms is smaller and the critical point reached earlier (see Fig. 3). Thus crowding of cells and decreased concentration of nutriment produce the same effect.

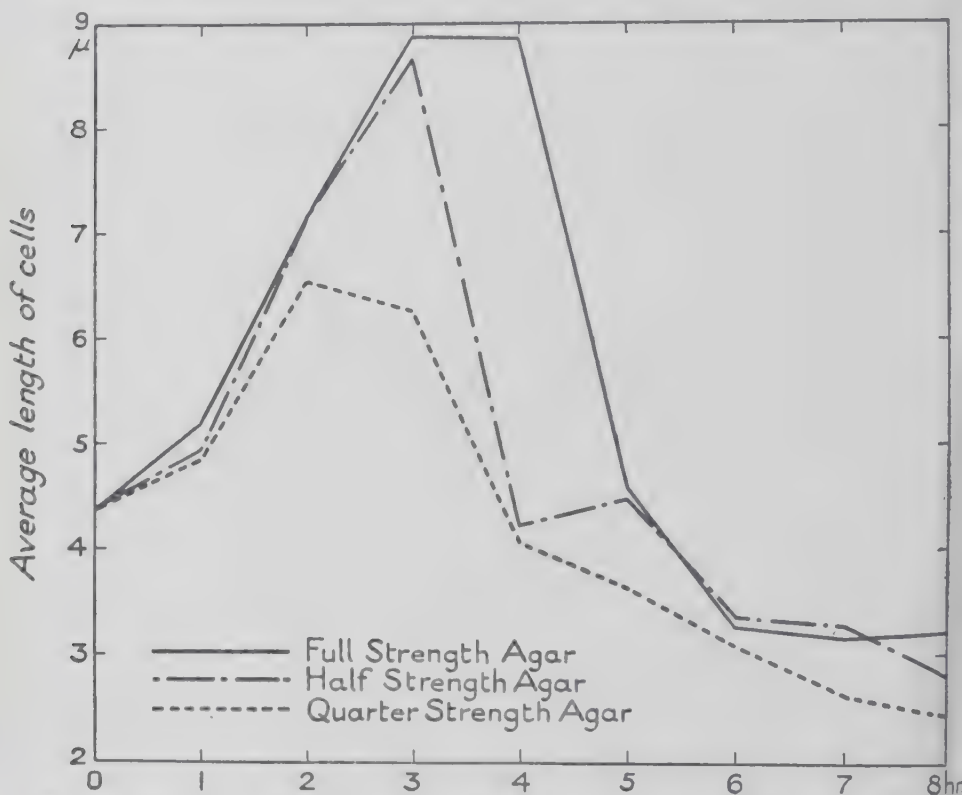


FIG. 3.—Influence of concentration of nutrients on average size of cells of *B. megatherium*¹

Hershey and Bronfenbrenner applied Henrici's initial observation to the study of the lag phase.² These workers followed the bacterial development through a 22-hour period by the viable count and by the estimation of total nitrogen (Table 1).

TABLE 1³

Age of culture, hours	Viable count, V.C.	Nephelometric count, N.C.	Nitrogen per ml., N	Apparent size, NC/VC	Ratio, N/NC
24.0	× 10 ⁶ /ml. 940	× 10 ⁶ /ml. 835	mg. 0.25	0.9	0.30
3.5	310	739	0.25	2.4	0.34

¹ Henrici, *Proc. Soc. exp. Biol. and Med.*, 1923, **21**, 346.

² Hershey & Bronfenbrenner, 1937, 1938.

³ Hershey, 1939.

Fig. 4 shows the usual phenomenon of lag when cell numbers are plotted against time, but no lag when cell material as measured by total nitrogen is in question. This discrepancy is explained when the volume of the cell is considered, the period of lag corresponding to the presence of large cells and high generation time, and the logarithmic phase to the return to normal cell size and low generation time.

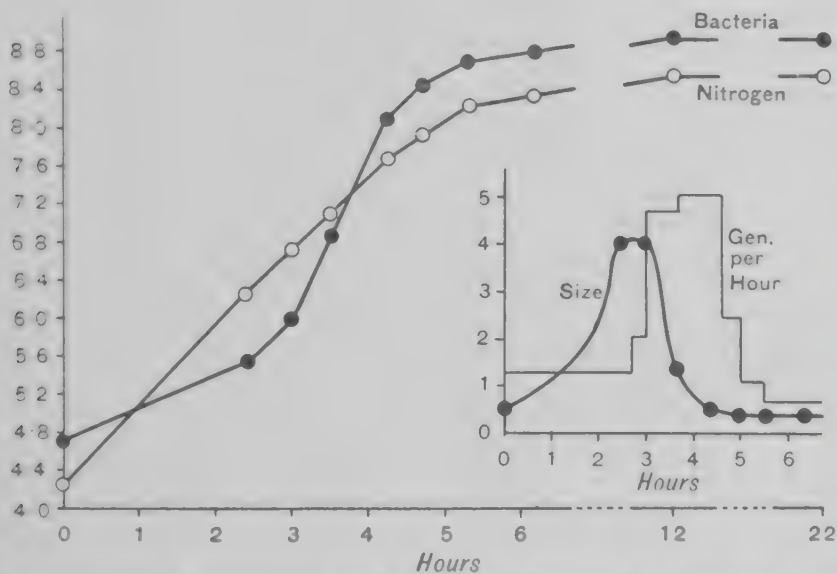


FIG. 4.—Increase of population and of bacterial nitrogen in *Bact. coli* culture. The logarithms of numbers and of nitrogen in mg. $\times 10^{10}$ per ml. are plotted against time. The insert shows the changes in average size expressed as nitrogen in mg. $\times 10^{10}$ per cell, and in the average rate of growth as reciprocal of generation time during the intervals observed¹

From the above observations it appears that a new definition of lag is needed, viz. (that phase of growth during which cell multiplication is retarded though increase in cell material may be occurring at the maximum rate.) This new view of lag makes the discussion of much work on that phase very difficult since it is probable that much of it is based on a misconception, whilst it is not certain that some instances of lag may not involve retarded growth as well as cell division. With this uncertainty in mind some characteristics of the phase may now be considered.

Early observations showed that the older the culture from which the inoculant is taken the longer the lag, and this has received recent corroboration. (It must be noted, however, that Hershey² has shown that this form of lag relates to cell division and not to growth.) Table 2 shows that in the early period of a culture sown from a 24-hour inoculation there are 0.7 generations per hour,

¹ Hershey, *Proc. Soc. exp. Biol. and Med.*, 1938, **38**, 128.

² *Ibid.*, 1939.

whilst in the corresponding period of a culture sown from a 3-hour inoculant there are 3·4 generations per hour, the rates of growth as measured by increase of total nitrogen being unaffected.

TABLE 2¹

Parent culture		Subculture								
Age, hours	Viable count, cells/ml.	Period of incubation, hours	Viable count, $\times 10^{-6}$ /ml.		Nitrogen, $\times 10^{-4}$ mg/ml.		Nitrogen, $\times 10^{-10}$ mg/cell		Rate of multiplication, gen/hr.	Rate of growth, twofold increase N/hr.
			Initial	Final	Initial	Final	Initial	Final		
3	7.4×10^6	1.50	0.37	51.0	0.49	14.0	1.32	0.28	3.4	3.2
24	2.2×10^9	1.58	2.20	58.0	0.57	21.0	0.26	0.36	0.7	3.3

TABLE 3²

INFLUENCE OF AGE OF INOCULANT ON RATES OF MULTIPLICATION AND GROWTH

Age of culture, hours	Rate of multiplication, gen/hr.	Rate of growth, twofold increase N/hr.
3.0	3.4	3.2
3.3	3.3	3.0
3.0	3.5	3.3
3.0	3.4	2.8
3.3	3.3	3.0
24.0	0.7	3.3
24.0	0.8	2.5
36.0	1.2	2.9
36.0	1.2	2.8
36.0	2.5	2.4
42.0	1.7	2.4

The type of lag due to age of inoculant is illustrated in an experiment of Salter³ in which subcultures were made at the end of 8 hours and the generation time compared with that of the parent culture (Table 4). If inoculations were made from older cultures the generation time in the initial phase was much prolonged (Table 5).

TABLE 4⁴

Time, hours	Average number of bacteria per c.c.	Generation time in minutes
Culture, <i>B. coli communis</i>		
0	305	—
4	9,450	51.5
8	2,570,000	31.5
Sub-culture		
4	945	—
8	238,500	31.9

¹ Hershey, *J. Bact.* (1939), **37**, 290.

² *Ibid.*, 1939.

³ Salter, 1919.

⁴ *Ibid.*, *J. Inf. Dis.* (1919), **24**, 260.

TABLE 5¹

Time, hours	Age of culture from which inoculations were made	Average number of bacteria per c.c.	Average generation time in minutes
	Days		
0	I	388	
2		1,070	72.1
4		13,000	33.3
6		177,000	31.8
8		4,400,000	25.9
0	4	349	
2		520	179.7
4		7,500	26.9
6		167,000	23.1
8		1,750,000	30.5

Highly instructive in this connection are the experiments of Sherman and Albus.² These workers showed that "old" cultures of *Bact. coli*, i.e. those over a week old, which had ceased for some days from appreciable multiplication, were markedly less sensitive to unfavourable conditions, such as exposure to cold (2° C.), heat (53° C.), 2% sodium chloride or 5% phenol, than were those taken from rapidly growing cultures a few hours after reinoculation. This was shown by removing samples from the culture and making a viable count (1) immediately, and (2) after exposure for 1 hour to the unfavourable condition. An example of their results is shown in Table 6.

TABLE 6³

EFFECTS OF EXPOSURE TO 2% NaCl UPON MATURE AND YOUNG CELLS OF *Bact. coli*

Exp. No.	Age of culture	Temperature incubated	No. of Bacteria per c.c.	
			At beginning	After 1 hour
1	21 hours	37° C.	890	770
2	2 days	Laboratory	378	356
3	7 "	"	1470	1410
4	7 "	"	880	960
1	3½ hours	37° C.	2530	27
2	3½ "	"	1110	72
3	3½ "	"	2190	55

The direct bearing of these results on the problem of the lag phase is displayed clearly in an experiment in which an old peptone culture of *Bact. coli* was sown into a new peptone medium and samples withdrawn at intervals and counted (1) direct, (2)

¹ Salter, *J. Inf. Dis.* (1919), 24, 260.

² Sherman & Albus, 1923 and 1924.

³ Ibid., *J. Bact.* (1923), 8, 127.

after exposure for 1 hour to 5% sodium chloride. Here the bacteria exhibit a decreasing tolerance for the salt solution as the lag phase advances, showing that they are undergoing a slow physiological change either of permeability or other surface condition evidenced by their reaction to the salt solution.

It has frequently been asserted that cells in the later stages of lag are in a high state of metabolic activity in respect of O_2 uptake, CO_2 elimination, deamination, etc.^{1, 2, 3} It is now clear that this is true only of chemical or metabolic activity related to cell numbers and disappears if related to quantity of cell material.⁴ This

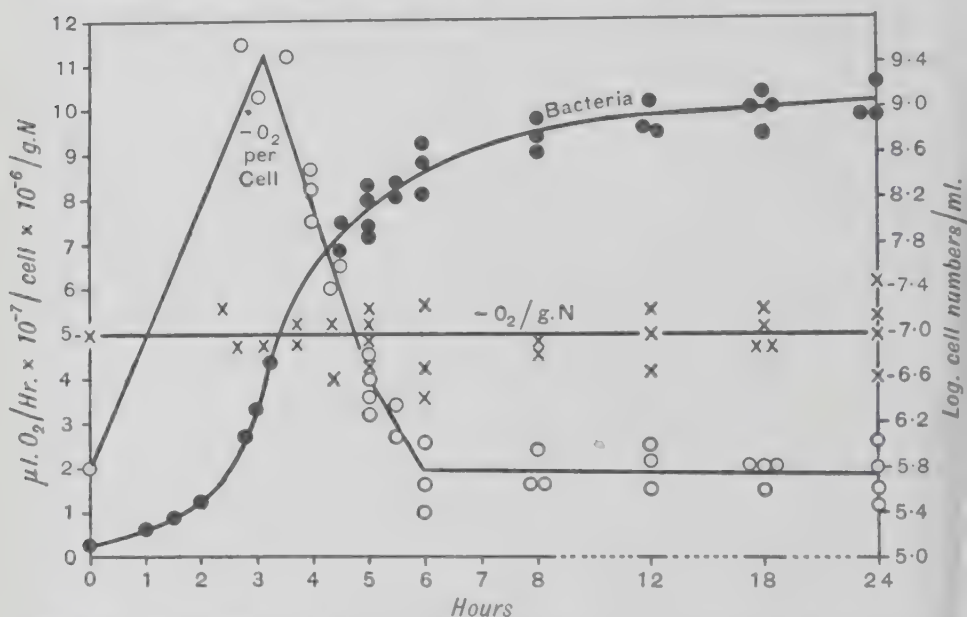


FIG. 5.—Rates of oxygen uptake per viable cell and per g. bacterial N in relation to phase of growth of *Bact. coli* cultures⁵

is shown in Fig. 5 in respect of respiratory activity and in Fig. 6 where rate of disappearance of glucose is shown to be strictly proportional to increase of cell material throughout the growth period.

Prolonged lag, actually a prolonged stationary phase, has been shown to occur when growth takes place in a synthetic as opposed to a broth medium.⁶ This effect is not attributable to change of medium and it is worthy of remark that the subsequent growth rate is not affected. It has been suggested that the effect is due to traces of poisonous metals which are in time removed by adsorption on to the colloidal particles introduced with the in-

¹ Martin, 1932.

² Walker & Winslow, 1932.

³ Walker *et al.*, 1934.

⁴ Hershey & Bronfenbrenner, 1937.

⁵ *Ibid.*, *J. Gen. Physiol.*, **21**, 726 (1937.)

⁶ Monod, 1942.

oculum; in broth media, well furnished with colloids, this removal occurs more quickly. This explanation is supported by the observation that the length of lag in brain broth was increased from 30 minutes to 2 hours by the presence of a strip of silver foil, the subsequent rate of growth and total crop being unaffected.¹

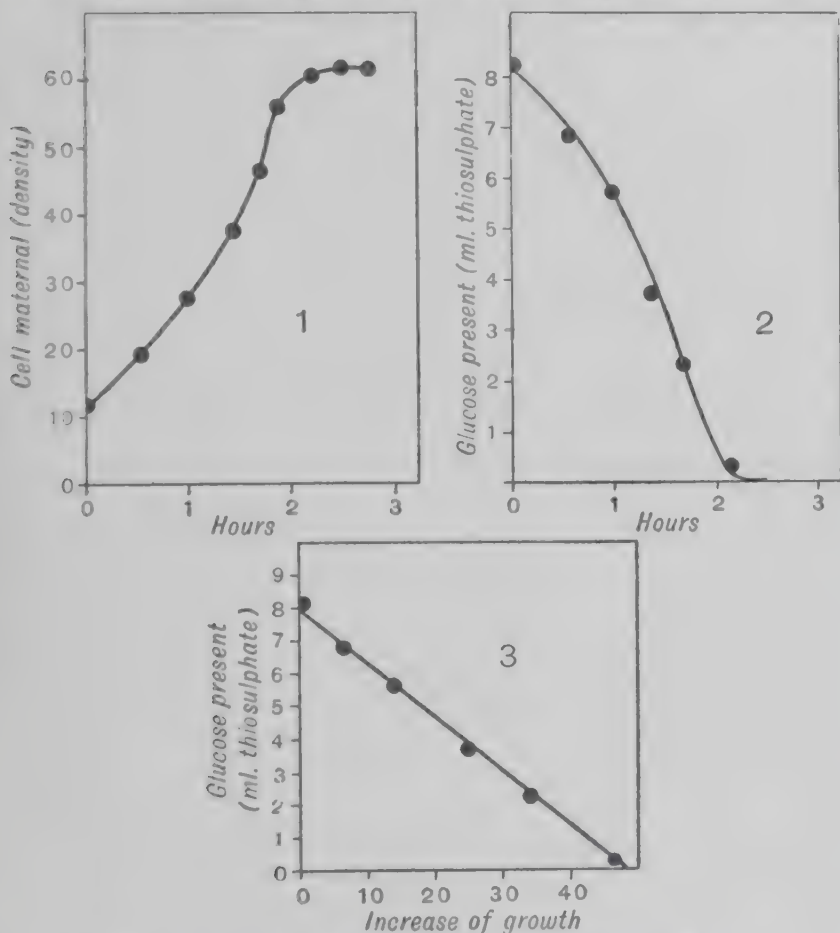


FIG. 6.—*Bact. coli* in synthetic media and glucose²

1. Rate of growth. 2. Consumption of glucose as function of time. 3. Consumption of glucose as a function of increase in growth.

In a synthetic medium the length of lag is decreased if the number of cells in the inoculant is increased; this may be due to adsorption of metal poisons as suggested above.

The relation of the viable to the total count

Another aspect of growth rate has been disclosed by the careful studies of Wilson.³ Working with *B. suispestifer* in a tryptic

¹ Monod, 1942.

² Ibid., *La Croissance des cultures bactériennes* (Hermann & Cie, Paris).

³ Wilson, 1926.

broth, he made both total and viable counts, and found that even during the logarithmic phase the total count exceeded the viable ; an example of his results is given in Fig. 7 and Table 7. These

LOG. OF
COUNT.

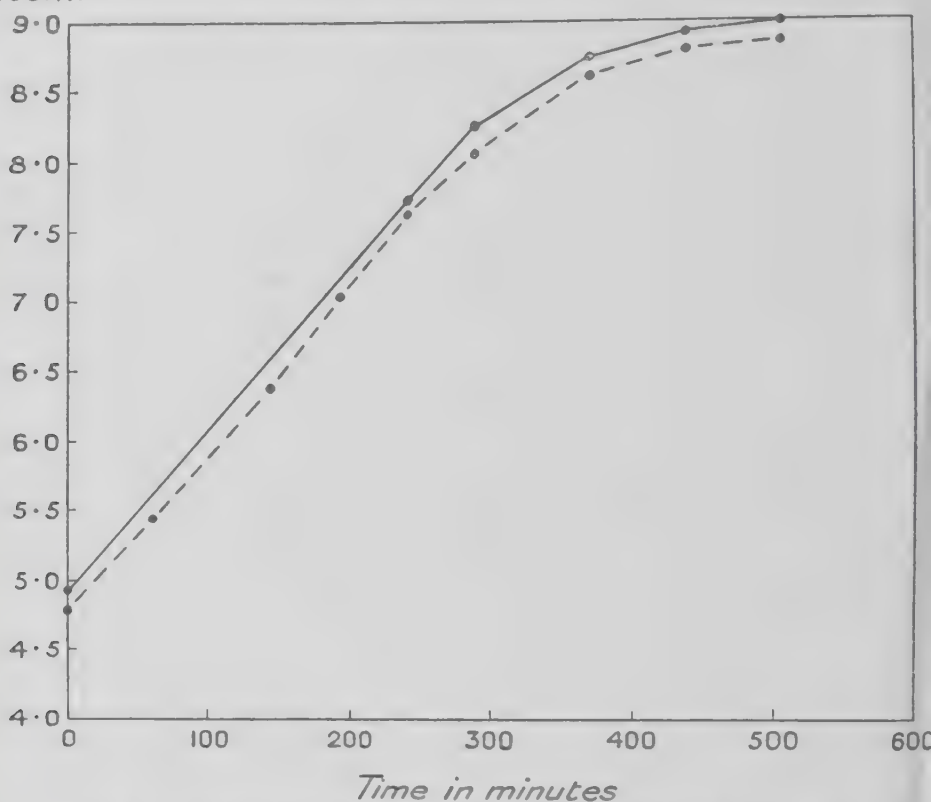


FIG. 7.—Showing the total and viable counts in a broth culture of *Bact. suispestifer*¹

Total count = continuous line. Viable count = interrupted line

TABLE 7²

Time after inoculation, minutes	Viable count per c.c.	Total count per c.c.	Relation of viable to total
0	62,150	81,470	per cent 76.28
60	249,700	—	—
140	2,417,000	—	—
190	10,570,000	—	—
240	43,290,000	50,780,000	85.23
290	116,900,000	176,000,000	66.37
370	416,100,000	535,100,000	77.76
440	646,800,000	860,400,000	75.16
510	781,500,000	1,045,000,000	71.90

¹ Wilson, *J. Bact.*, 1922, 7, 434.

² Ibid.

results agree with the theory that in every generation the majority of the organisms continue to divide, whilst a constant percentage fail to do so. To make this clear, Wilson supposes 1000 organisms per c.c. alive at the beginning of the logarithmic phase; at the end of the first generation there would be 2000 organisms, of which, say, 80%, or 1600 would live and divide, whilst 20% or 400 would die. The continuation of the process is seen clearly in Table 8.

TABLE 8

	Viable	Organisms per c.c.	
		Non-viable	Total
Start	1,000	0	1,000
End of 1st generation	1,600	400	2,000
" 2nd "	2,560	640 + 400 = 1,040	3,200 + 400 = 3,600
" 3rd "	4,096	1,024 + 1,040 = 2,064	5,120 + 1,040 = 6,160
" 4th "	6,555	2,064 + 1,637 = 3,701	8,192 + 2,064 = 10,256
" 5th "	10,488	3,701 + 2,622 = 6,323	13,110 + 3,701 = 16,811
" 6th "	16,781	6,323 + 4,195 = 10,518	20,976 + 6,323 = 27,299

If the logarithms of these counts are plotted against the time, the curve for the viable organisms lies along an oblique straight line, whereas that for the total rises at first slightly more rapidly and then continues along an almost straight line very slightly divergent from that of the viable count (Fig. 8).

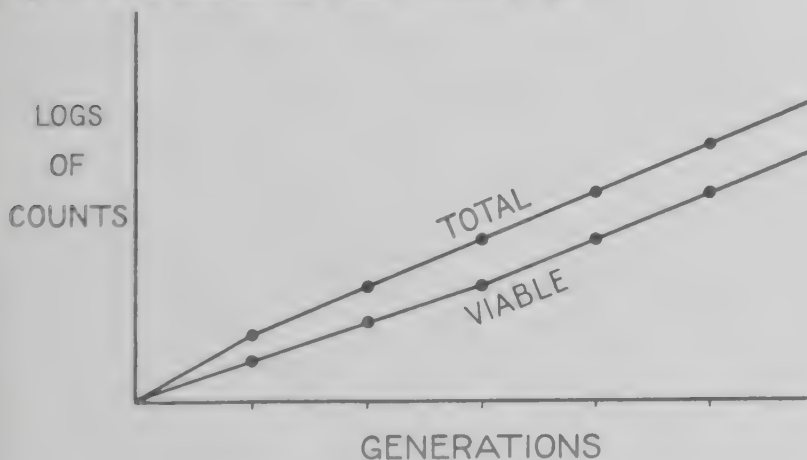


FIG. 8

It was pointed out by Wilson that this view of the discrepancy between the total and viable counts involves a modification in our method of calculating the generation time of an organism. According to the assumption that during the logarithmic phase all the organisms produced in each generation are viable, then the number of generations in a given period is calculated from the formula $n = \frac{\log b - \log a}{\log 2}$, where n = the number of generations,

b the number of organisms at the end, and a the number at the beginning of the given period. But if (say) 80% only are dividing the number of organisms in each generation rises by 1.6 instead of

2, hence the formula required is $n = \frac{\log b - \log a}{\log 1.6}$, which de

creases the figure for the average generation time. Thus Wilson calculates from one of his experiments a generation time of 25.5 minutes, according to the old formula, and of 19.7 minutes according to the new.

Causes affecting reproductive rate

The rate of reproduction is largely influenced by temperature. Thus measurable reproduction in *Bact. coli*, for example, begins at

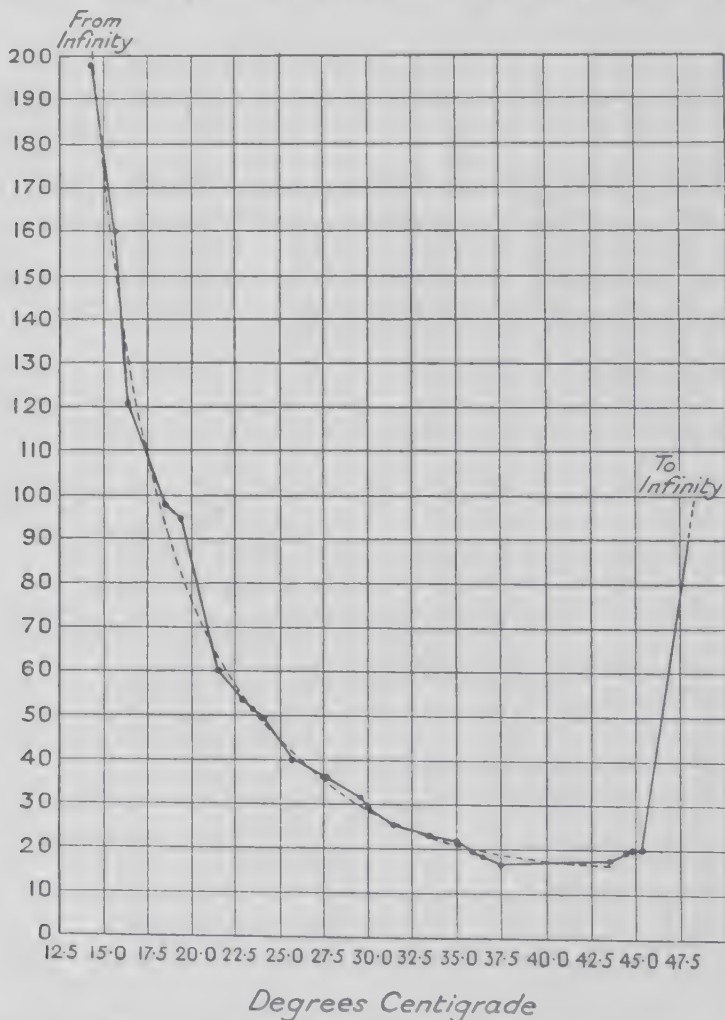


FIG. 9.—The growth rate of *Bact. coli* at different temperatures¹

¹ Barber, *J. inf. Dis.*, 5, 396 (1908).

out 10^8 and increases rapidly to 37° , where it reaches a maximum of a generation time of 17 minutes; this remains nearly constant to 45° , when it falls rapidly till growth practically ceases at 49° (Fig. 9). The rates of growth of *Bact. coli* on synthetic media with glucose, mannitol, sorbitol and maltose are very close; the Q_{10} is given in Table 9. The log of the growth rate plotted against the reciprocal of the absolute temperature is shown in Fig. 10.

TABLE 9

Source of carbon . .	Q_{10}	
	$23^\circ-33^\circ$	$27^\circ-37^\circ$
Glucose	2.1	1.90
Mannitol	2.0	1.85
Sorbitol	2.1	1.90
Maltose	2.1	1.80

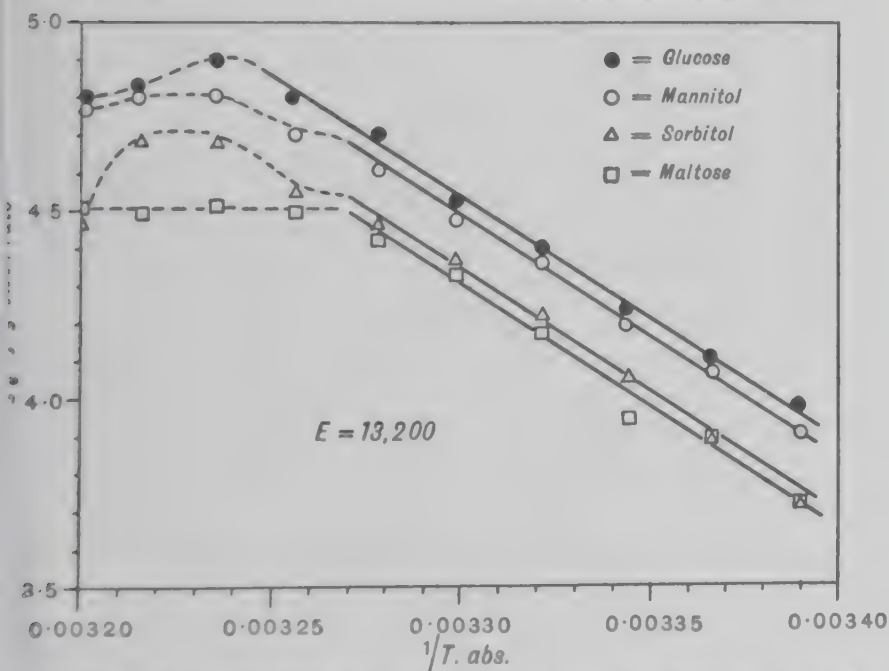


Fig. 10.—*Bact. coli* on synthetic medium with different sources of carbon. Log of the growth rate as a function of the reciprocal of the absolute temperature¹

The effect of pH

The effect of pH on multiplication rate is very marked but varies with different organisms. Much published work on this subject is vitiated because it takes account only of the initial pH. In some

¹ Morosel (1942), *La Croissance des cultures bactériennes* (Hermann & Cie, Paris).

media, e.g. those containing fermentable carbohydrate, this changes so rapidly that the pH at which the reproduction rate is measured is far removed from that at the start. Fig. 8, Chapter XI, shows that *E. coli* grows between pH (initial) 4.5 and 9.0. Table 10 shows how far these values vary during the course of the experiment.¹

TABLE 10
VARIATION OF pH OF MEDIA (TRYPTIC DIGEST OF CASEIN)
DURING GROWTH OF *E. coli*

Buffer	pH		
	Initial	Final	Mean
M/60 phthalate . .	4.55	4.71	4.63
M/60 phthalate . .	4.95	5.58	5.26
M/45 phosphate . .	6.16	6.58	6.37
M/45 phosphate . .	7.01	7.10	7.05
M/45 phosphate . .	8.21	7.82	8.01
M/60 borate . . .	8.82	8.70	8.75
M/60 borate . . .	9.10	9.10	9.10

Effect of food concentration on growth rate

The rate of growth in the logarithmic phase is largely independent of concentration of carbon nutrients until this reaches a low level. This has been shown in brain broth and also in synthetic medium for both *coli* and *subtilis*. In the former the minimum generation time for *subtilis* is 36 minutes (see Table 11), in synthetic medium with sucrose 45 minutes, whilst *coli* on glucose attains a generation time of 45 minutes. Fig. 11 shows the influence of glucose concentration on generation time for *coli*. Maximum growth rate is reached at about 25 mg./litre, half-rate

TABLE 11
Bac. subtilis ON BRAIN BROTH. RATE OF GROWTH, ETC., IN
THE LAG PHASE AS A FUNCTION OF THE INITIAL
CONCENTRATION OF THE MEDIUM

Initial conc. of medium $\times 0.005$ g./ml.	Rate of growth No. divs./H	Gen. time, minutes
15.0	1.67	36
12.0	1.65	36
8.0	1.65	36
5.0	1.64	37
4.0	1.43	42
3.0	1.18	52
1.5	0.87	69

¹ Gale & Epps, 1942.

at 4 mg./litre. The resemblance between this curve and one in which enzyme activity is plotted against concentration of substrate is obvious and indicates that growth rate may be controlled by the rate of action of one enzyme in the chain of glycolytic reactions; this idea has been critically discussed by Monod.

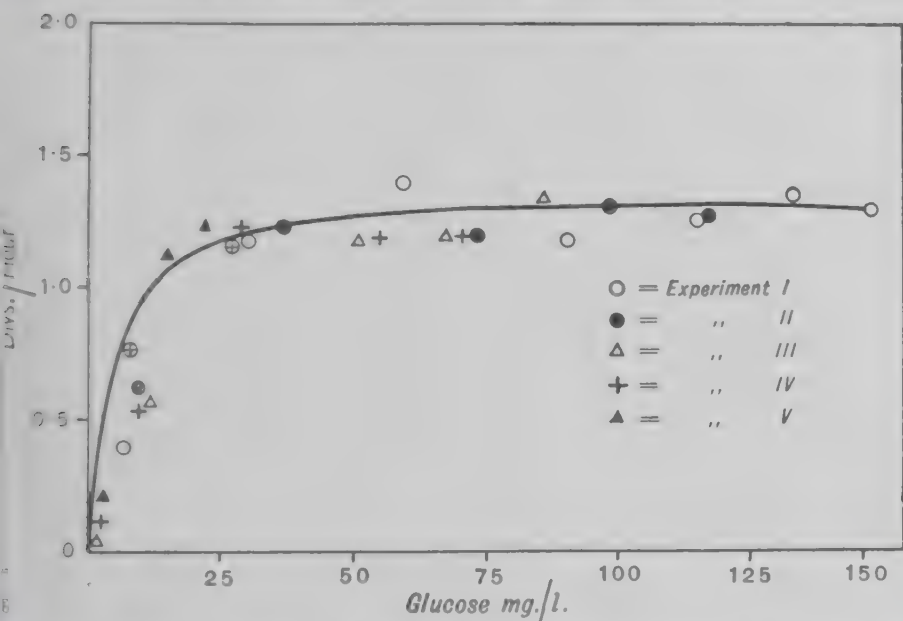


FIG. 11.—Rate of growth of *Bact. coli* in synthetic medium as a function of the concentration of glucose¹

Factors influencing total crop

In studying the factors influencing the total bacterial substance produced on any given media the problem of aeration becomes very important² where aerobes or facultative anaerobes are in question. There is little doubt that insufficient aeration has, in the past, often been the limiting factor when cessation of growth has been attributed to other causes. Mechanical agitation—as in a Warburg tank—suffices if the depth of the medium does not exceed 8 mm.; when this is exceeded bubbling sterile air from a pressure cylinder through a porous glass filter or similar device must be employed. The necessity for adding CO₂ in the early stages must also be emphasised; CO₂ as a growth factor has been discussed elsewhere (pp. 86 and 213). The accompanying graph³ (Fig. 12) shows the relation of CO₂ to growth rate. The amount of bacterial growth is usually measured by a photo-electric turbidimeter. The relation of this to cell material and also to cell numbers once

¹ Monod, 1942, p. 76.

² Ibid. 1942.

³ Dagley & Hinshelwood, 1938.

the logarithmic phase is reached has been tested by a number of workers and may now be taken for granted.¹

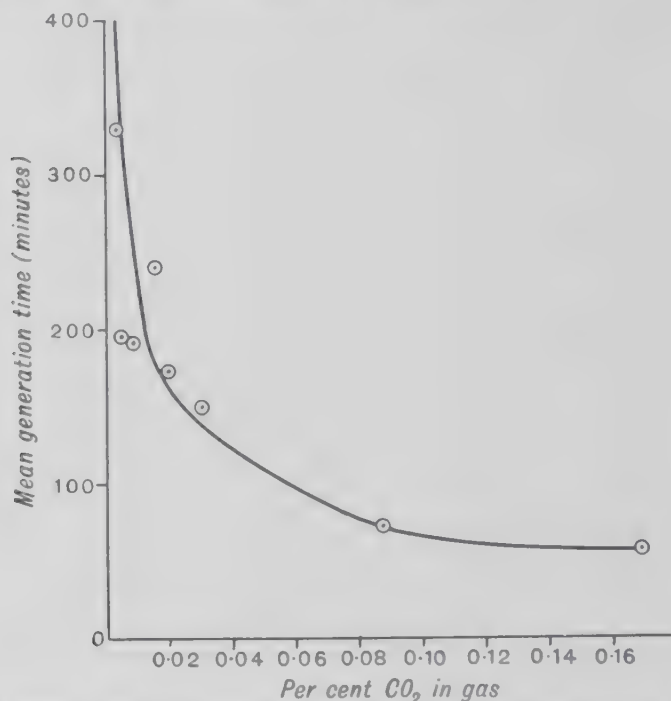


FIG. 12.—Effect of concentration of CO₂ on the growth rate of *Bact. lactaerogenes*²

The relation of total crop to concentration of medium has been tested out by Monod.³ It is important to select for this purpose an organism which can grow on a simple medium in the absence of growth factors and amino-acids, otherwise absence or short supply of any of these may become limiting. Using *coli* growing on brain broth, the total crop was shown to be strictly proportional to the concentration of the medium between 0.005 and 0.1 g./ml. (wet weight). (The units in which the crop was measured are not supplied.) A similar result was obtained with *Bac. subtilis*. It was shown in both cases that the limiting factor was carbohydrate supply, glucose added after growth had ceased causing its immediate resumption, whilst added NH₄Cl or phosphate had no effect.

Studies of both *coli* and *subtilis* on synthetic media showed that the crop is strictly proportional to the concentration of glucose or sucrose between 0.2 and 0.02 mg./ml., the mean value for the ratio mg. bacteria (dry) per mg. carbohydrate being 0.233 in the case of *coli* and glucose and 0.218 for *subtilis* and sucrose. N

¹ Monod, 1942, p. 38.

² Dagley & Hinshelwood, *J. Chem. Soc.*, 1938.

³ Monod, 1942, p. 38.

tendency was found for this ratio to alter as the concentration of the carbohydrate fell off, and the author concludes that the sole factor limiting crop is the amount of carbon material, and that the ratio of crop to concentration of medium is independent of the dilution of the food material. This implies that there exists no demand for maintenance apart from growth. Thus if M = total crop, C the concentration of food and C_0 the concentration required for maintenance apart from growth, then $M = K(C - C_0)$ and C_0 is the point where the straight line cuts the abscissa. But since in all cases tested the line passes through the origin it may be deduced that C_0 is 0 and hence that the sole use of the carbon constituent of this medium is for growth, the conception of energy for maintenance becoming unnecessary.

TABLE 12¹TOTAL GROWTH OF *Bact. coli* ON SYNTHETIC MEDIUM + GLUCOSE

Conc. glucose, mg./litre A	Total growth, units of density	Total growth, mg. dry wt. per litre B	Ratio $\frac{B}{A}$	Error % digression from mean
200	58.7	47.0	0.235	+ 0.9
180	53.0	42.4	0.236	+ 1.3
160	46.8	37.4	0.234	+ 0.43
140	41.3	33.0	0.236	+ 1.3
120	35.0	28.0	0.234	+ 0.43
90	26.0	20.8	0.230	- 1.3
70	20.0	16.0	0.228	- 2.1
50	14.4	11.5	0.230	- 1.3
25	6.7	5.4	0.258	+ 10.7
			Mean 0.233	

TABLE 13²TOTAL GROWTH OF *Bac. subtilis* ON SYNTHETIC MEDIUM + SUCROSE

Conc. sucrose, mg./litre A	Total growth, units of density	Total growth, mg. dry wt. per litre B	Ratio $\frac{B}{A}$	Error % digression from mean
300	82.5	61.0	0.203	- 6.9
250	68.0	54.4	0.218	0.0
200	56.5	45.2	0.225	+ 3.2
150	38.3	30.6	0.204	- 6.4
100	26.2	21.0	0.210	- 3.7
50	15.5	12.4	0.248	+ 13.7
25	8.0	6.4	0.256	+ 17.4

¹ Monod, 1942, p. 44.² Ibid., p. 43.

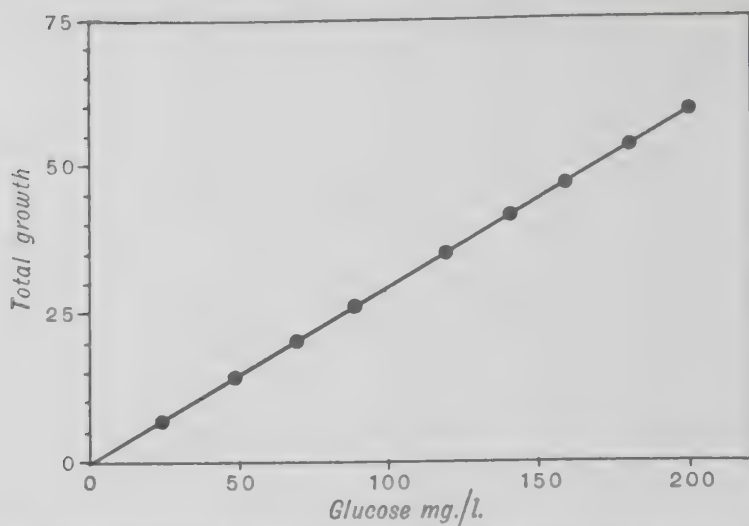


FIG. 13.—Total growth of culture of *Bact. coli* in synthetic medium as a function of the concentration of glucose¹

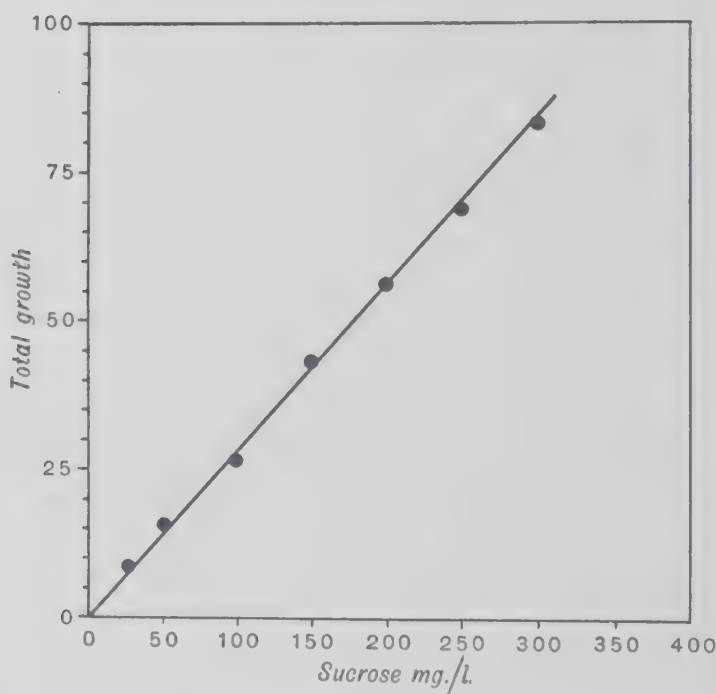


FIG. 14.—Total growth of culture of *Bac. subtilis* in synthetic medium as a function of the concentration of sucrose²

¹ Monod (1942), *La Croissance des cultures bactériennes* (Hermann & C Paris).

² Ibid.

The effect of growth rate on crop

It has been shown that in synthetic media in optimal conditions bacterial crop is proportional to the concentration of the carbon compound and that the rate is independent of concentration within wide limits. If now the growth rate is limited by some factor such as suboptimal oxygen supply or change of pH , it is found that the total crop is unaffected, though of course taking longer to attain. This is true both for synthetic and complex media¹ (see Fig. 15). These results provide additional evidence against the idea that the cell requires energy for maintenance apart from

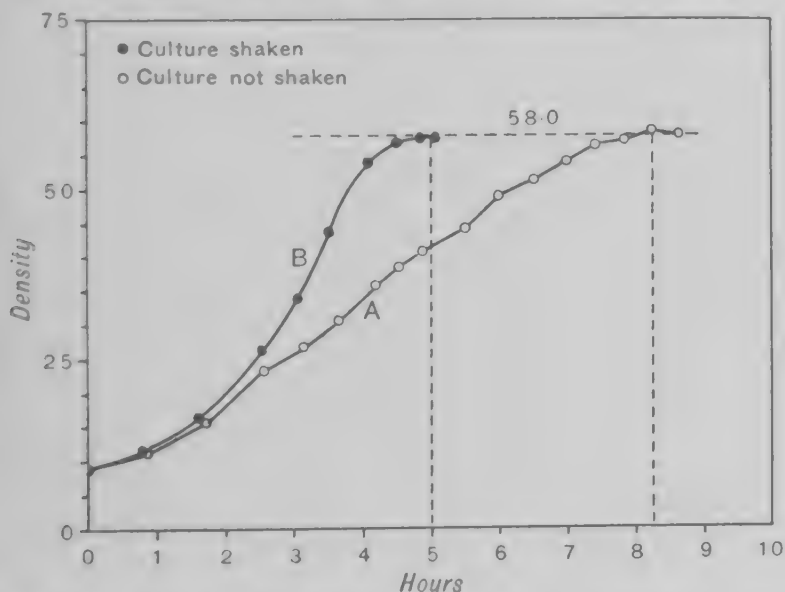


FIG. 15.—Growth of two cultures of *Bact. coli* on synthetic medium + ammonium lactate. A at rest, B shaken²

growth. Such energy is proportional to t , the time of the growth period, and D , the amount or density of living material produced in time t . Since D is the same whether $t = 5$ or 8 hours, the evidence that the cell requires some part of the energy derived from the food material for purposes other than growth falls to the ground. For further discussion of this subject see Monod.³

Influence of temperature on crop

The influence of temperature on crop is shown in Fig. 16. For the four carbon sources given, between 29° and 33° the crop is unaffected by temperature, between 33° and 41° it falls off and

¹ Monod, 1942.

² *Ibid.* (1942), *La Croissance des cultures bactériennes* (Hermann & Cie, Paris).

³ *Ibid.*, 1942, p. 92.

between 29° and 23° it rises. Closely parallel results are obtained for the four compounds tried.

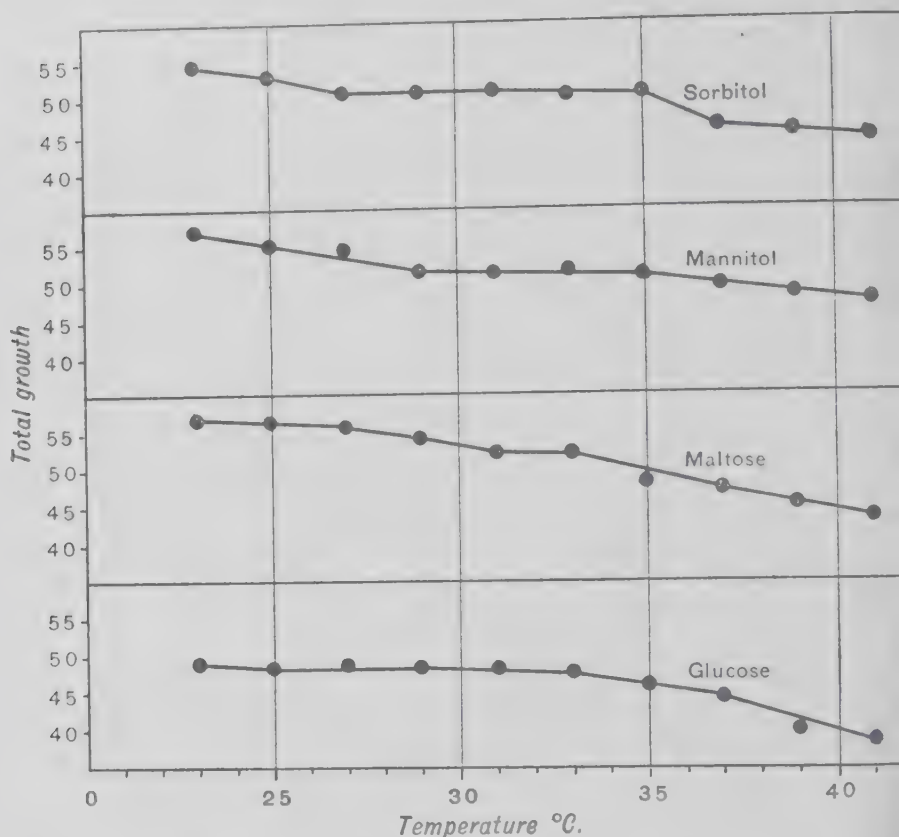


FIG. 16.—Total growth as a function of temperature. *Bact. coli* on synthetic medium in presence of different carbon sources¹

NUTRITION

General considerations

The food requirements of bacteria differ profoundly from species to species. At one end of the scale are the strict autotrophs whose needs are met by inorganic materials, carbon being supplied in the form of carbonate or carbon dioxide and nitrogen as ammonium, salts, nitrates or nitrites. At the other end of the scale are organisms which can be grown at present only in complex protein digests to which are added blood or other tissues, culminating in such organisms as *M. lepræ*, which has so far defied all efforts at cultivation *in vitro*.

¹ Monod (1942), *La Croissance des cultures bactériennes* (Hermann & Cie Paris).

Salt requirements

The basal salt requirements common to all bacteria are unknown. In the absence of exact information the following are usually supplied and found adequate: Na, K, Mg, Fe, SO_4 , PO_4 and Cl.

In the case of *E. coli*¹ a study with carefully purified salts using a nutrient broth as control for optimum requirements gave the following results: The basal salts used were NaCl 0.5% (NH_4) $_2\text{SO}_4$ 0.8%, KH_2PO_4 0.2% and Na_2HPO_4 0.2%. Fe and Mg were both necessary for optimum growth, exerting their maximum effect at 0.5 $\mu\text{g. ml.}$ and 0.5–1.4 $\mu\text{g. ml.}$ respectively.

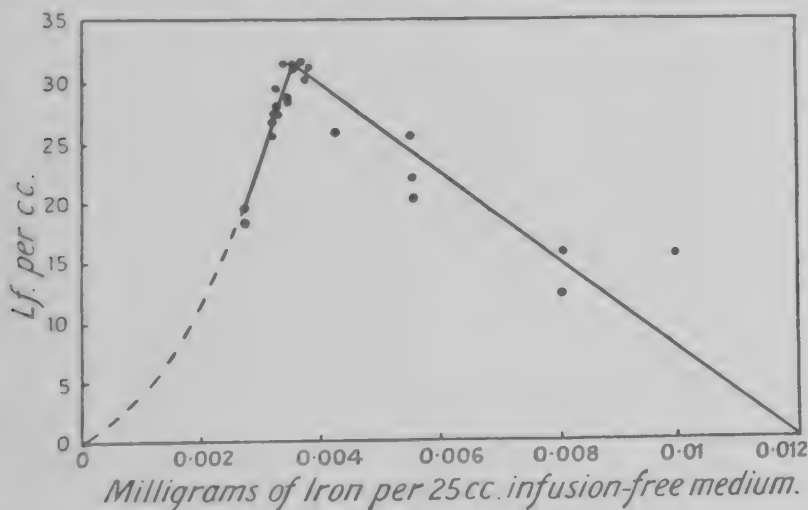


FIG. 17

The effect of Mg was detectable at 0.000005 $\mu\text{g./ml.}$ and Fe at 0.0005 $\mu\text{g. ml.}$ The following were found to exert no effect: Ca, Sr, Co, Ni, Mn, Zn, Al, Cu, Rb, Cs, Si and Mb.

It is probable, however, that other organisms have further requirements. Traces of Cu and Zn have been found to improve the growth of *C. diphtheriae*² and the effect of Mb on nitrogen fixation is mentioned on p. 232. Another striking case is that of *C. diphtheriae*³ in which toxin production on laboratory medium is extremely sensitive to the concentration of iron, $2.8 \times 10^{-6} M$ being optimum with a sharp fall on either side. The inhibitive effect of increased concentration is on toxin production, not on growth; experimental data at low concentrations of iron (dotted line) are lacking (Fig. 17). See, however, recent work, Chapter V, p. 120.

A possible method for determining the inorganic requirements

¹ Young *et al.*, 1944.

² Happold *et al.*, private communication.

³ Pappenheimer & Johnson, 1936.

of any strain of bacteria might be the analysis of the ash. This, however, provides no safe guide since the inorganic content of the bacterial cell differs enormously not only from species to species and from strain to strain but also according to the inorganic content of the medium on which the organism is grown. These points are illustrated in Tables 14, 15 and 16.

TABLE 14¹
ORGANISMS GROWN ON SURFACES OF MEAT PEPTONE BROTH AGAR

Organism	g. of element per cent dry weight of organism					
	K	Mg	Ca	Na	P	Fe
<i>Bact. coli</i>	0.036	0.20	0.16	0.83	1.52	0.0175
<i>Bact. typhi</i>	0.22	0.27	0.14	0.92	1.52	0.0075
<i>Bact. aertrycke</i> (rough)	0.065	0.35	0.19	0.79	1.82	0.01
<i>Bact. aertrycke</i> (smooth)	0.022	0.12	0.184	0.74	1.65	0.005
<i>Staph. albus</i>	0.62	0.20	0.136	0.41	1.68	0.0075
<i>Staph. aureus</i>	0.64	0.13	0.11	0.27	1.30	0.0105
<i>Bac. anthracis</i> (rough) .	0.64	0.32	0.10	0.86	1.52	0.0066
<i>Bac. anthracis</i> (smooth)	0.28	0.28	0.03	0.82	1.38	0.0066
<i>V. cholerae</i> (rough) . .	0.076	0.31	0.094	0.76	1.21	0.008
<i>V. cholerae</i> (smooth) . .	0.048	0.15	0.076	0.76	1.21	0.003

TABLE 15²
VARIATIONS IN THE ASH CONTENT OF *B. coli*

Water	Lowest per cent	Highest per cent
Water	60.2	79.6
Ash	2.11	7.83
P ₂ O ₅	0.92	4.22
CaO	0.04	2.71

TABLE 16³
INFLUENCE OF SALT CONTENT OF MEDIUM ON ASH CONTENT OF BACTERIA

Vibrio cholérique (Past.), var. "smooth"	Ash, per cent	K per cent		Na per cent		Ca per cent	
		Medium	Bact.	Medium	Bact.	Medium	Bact.
Medium I	6.02	0.445	0.402	6.0	0.76	0.066	0.13
„ II	5.10	0.69	0.048	5.40	0.76	0.025	0.076
		Mg per cent		Fe per cent		P per cent	
		Medium	Bact.	Medium	Bact.	Medium	Bact.
Medium I	6.02	0.245	0.09	0.039	0.08	1.0	1.48
„ II	5.10	0.13	0.15	Traces	0.003	0.87	1.21

Guilleman and Larson,⁴ studying *Bact. coli*, found that on death by heat about half of the salts diffuse out, whilst the other

¹ Damboviceanu & Barber, 1931.

³ Damboviceanu & Barber, 1931.

² Dawson, 1919.

⁴ Guilleman & Larson, 1922.

half remain fixed in the cell and contribute to the analysis of the cell ash. Whether we are justified in considering the former as non-essential and accidental and the latter as essential components of the cell is doubtful, but the observation serves to explain the large differences in ash content noted by previous observers, according to the type of medium on which the cell is grown. The results of Guilleman and Larson are summarised by Falk¹ as follows:

Total dry matter in organisms	11.34%
Total ash in the dry matter	12.75%
Fixed salts in the ash	42.79%
Free salts in the ash	57.21%

The high ratio of K : Na and the confining of iron to the non-diffusible constituents of the cell are in harmony with analysis of other cells. There is every probability that the figures for the fixed salts of the ash in Table 17 represent a rough picture of bacterial inorganic composition, but doubtless special constituents

TABLE 17²

Element or group	Fixed salts as per cent dry wt.	Free salts as per cent dry wt.
Cl	0.00	7.40
Ca ₃ (PO ₄) ₂	35.61	23.59
CaO	13.77	9.13
P ₂ O ₅	21.84	14.46
MgO	5.92	2.04
SO ₄	1.78	4.36
Fe ₂ O ₃	3.35	Trace
K	12.95	9.94
Na	2.61	19.77
P ₂ O ₅	33.99	26.84
Carbon	—	1 +
Total	96.21	96.94

enter into the composition of certain organisms. It will be noted that the elements of the "fixed" salts find a place in most inorganic media.

General nutritional requirements

All autotrophic bacteria supply the carbon and nitrogen requirements of the growing cells from carbon dioxide and ammonia. These organisms have the simplest requirements and the most complex synthetic machinery, and are independent of other forms of life for any constituent of their food.

¹ Falk, *Abstr. Bact.* (1923), 7, 33.

² Ibid.

A number of heterotrophants can use ammonia as the sole source of nitrogen, the carbon being supplied by carbohydrate or simple organic acids. Thus in a nutritional study by Koser and Rettger,¹ out of 39 organisms tested 21 grew on a basal medium with glycerol and ammonium phosphate, from which these organisms are therefore able to synthesise all their amino-acids. No marked improvement in growth occurred when ammonia was replaced by single amino-acids or by mixtures of two or three (e.g. glycine, aspartic acid and tyrosine). With a complicated mixture containing, in addition to the three amino-acids mentioned, also valine, glutamic acid, lysine, histidine, tryptophan, besides taurine, creatine and allantoin, more rapid growth was obtained and the mixture also supported the growth of four organisms, viz. *Bac. subtilis*, *Bact. typhosum*, *B. pullorum* and *Sarc. lutea*, which did not grow on glycerol and ammonia or on a single amino-acid.

These observations find their explanation in later work, in which it is shown that certain organisms (e.g. some strains of *Bact. typhosum*) lack the power to synthesise tryptophan and, when this is added, are able to grow in media in which all other amino-acids are replaced by ammonium phosphate. Moreover, it is now clear that failure to grow on simple amino-acids or on mixtures of these may have nothing to do with failure to synthesise protein but to the absence of growth factors. Organisms able to grow on simple amino-acids as well as on ammonia probably actually use the latter obtained from the amino-acid by deamination. The more rapid growth on the complex mixtures may mean that synthesis of some of the amino-acids is a slow process and limits the growth rate, which increases if the amino-acid is supplied ready-made.

The optimum concentration of ammonia appears to be contained in 0.1% ammonium chloride, though growth capable of subcultivation is obtained in 0.01%.²

The use of the term "non-ammonia-assimilating" for organisms which fail to grow on ammonium salts without the addition of amino-acids is misleading and has led to much confusion of thought. The failure to grow may be due to inability to synthesise one or more of its amino-acids from ammonia and a simple carbon compound—as in the case of *Bact. typhosum* (see above)—or it may be due to the absence of some growth factor unconnected with protein synthesis.

Use of carbon compounds

Autotrophic bacteria obtain their carbon for growth from carbon dioxide or carbonate, so their carbon requirements need no further discussion. Heterotrophants are assumed to require carbon com-

¹ Koser & Rettger, 1919.

² Friedlein, 1928.

pounds for energy and cell synthesis ; whether energy is required for the bacterial cell, except for purposes of synthesis, has been already discussed. The variety of organic compounds available to an organism varies enormously from species to species and probably forms the principal basis of its adaptation to environment.

A valuable study on this subject by den Dooren de Jong¹ consists in showing which among a large number of compounds can serve as sole source of carbon in a synthetic medium for a group of common organisms which can use ammonia as sole source of nitrogen ; his results are given in Table 18. It is instructive to compare the utilisation of the amines, amino-acids, etc., as sources of carbon and as sources of nitrogen. These records show that carbohydrates and related compounds are the most generally utilised ; next come malic, citric, succinic and lactic acids, followed by the fatty acids and lastly by the monohydric alcohols. Where negative results are obtained throughout, as with *Microc. albus*, one suspects that growth fails for lack of an unknown growth factor rather than from complete inability to oxidise so representative a collection of compounds.

By the use of selective media organisms were isolated from the soil with much more varied oxidative powers. Thus, whereas in the group of stock organisms in Table 18 the amines were in general not available as sources of carbon, *Ps. aminovorans* α isolated from soil on media containing amines as source of carbon showed much enhanced powers of utilising these compounds. Similar examples from the same work could be given, from which one learns that there exist in the soil organisms with extremely varied oxidative mechanisms of which the strains in cultivation in the laboratory are very meagre representatives ; *Ps. putida*, for example, is able to supply its carbon requirements from 77 out of 200 compounds tested, including 6 carbohydrates, 10 alcohols, 10 saturated fatty acids, 3 unsaturated fatty acids, 17 amino-acids, 9 amides and 7 amines.

The question whether any given compound can function as a source of carbon for any given organism depends on several factors : (1) The organism must be able to decompose the compound, as it is unlikely that any constituent of media is sufficiently reactive to enter without change into the material of the cell. The change may occur aerobically or anaerobically by any of the methods discussed in Chapters II, IV and V, and must yield energy for subsequent synthetic reactions. (2) It must also yield some compound which can be utilised by the cell for synthetic purposes. Examples of substances readily oxidised but failing to support

[Continued on p. 192]

¹ den Dooren de Jong, 1926.

TABLE 18¹

Tapwater with 0.1% K_2HPO_4 , 0.1% Am_2SO_4 , 1% $CaCO_3$, 0.5% of the undermentioned compounds	<i>Bac. vulgatus</i>	<i>Bac. mycoides</i>	<i>Bac. polymyxa</i>	<i>B. aerogenes</i>	<i>B. coli</i>	<i>B. prodigiosum</i>	<i>B. herbicola</i>	<i>B. vulgare</i>	<i>Myc. phlazi</i>	<i>Microc. albus</i>	<i>Sarc. lutea</i>	<i>Ps. fluorescens</i>	<i>Ps. aminovorans</i> α
Formic acid . . .	—	—	—	—	—	—	—	(—)	—	—	—	(—)	
Acetic acid . . .	(—)	(—)	—	+	(—)	(+)	(—)	(—)	+	—	+	(+)	+
Propionic acid . . .	(—)	(—)	—	—	—	—	(—)	(+)	+	—	+	(+)	
Butyric acid . . .	(—)	(—)	—	(—)	—	—	(—)	(—)	+	—	+	(—)	
Isobutyric acid . . .	(—)	(—)	—	—	—	—	(—)	(—)	+	—	+	(+)	
Valeric acid . . .	(—)	(—)	—	—	—	—	(—)	(—)	+	—	+	—	+
Caproic acid . . .	(—)	(—)	—	(—)	—	(+)	(—)	(+)	+	—	+	+	
Heptylic acid . . .	(—)	—	—	—	—	(+)	—	(+)	+	—	+	+	
Caprylic acid . . .	—	—	—	—	—	+	—	—	+	—	+	+	
Nonylic acid . . .	—	—	—	—	—	+	—	—	(+)	—	+	+	
Capric acid . . .	—	(—)	—	—	—	+	—	—	(+)	—	(—)	+	
Lauric acid . . .	—	—	—	—	—	—	—	—	(—)	(—)	—	—	
Palmitic acid . . .	—	—	—	—	—	—	—	—	—	(—)	—	—	
Stearic acid . . .	—	—	—	—	—	—	—	—	—	(—)	—	—	
Acrylic acid . . .	—	—	—	(—)	—	—	—	—	—	—	—	—	
α -Crotonic acid . . .	—	—	—	—	—	—	—	—	+	—	(+)	—	+
Undecylic acid . . .	—	—	—	—	—	—	—	(—)	+	—	(+)	(—)	—
Oleic acid . . .	(—)	(—)	—	—	—	(+)	—	—	(+)	—	(+)	(+)	
Elaidic acid . . .	—	—	—	—	—	—	—	—	(—)	—	—	—	
Glycollic acid . . .	—	—	—	—	—	—	—	—	—	—	—	—	
Lactic acid . . .	(+)	—	—	+	+	+	+	—	+	—	+	+	+
α -Hydroxybutyric acid . . .	(—)	—	—	—	—	—	—	—	(—)	—	—	—	+
β -Hydroxybutyric acid . . .	—	—	—	—	—	(+)	—	—	(+)	—	(—)	+	+
Hydroxyisobutyric acid . . .	—	—	—	—	—	—	—	—	—	—	—	—	
Glyceric acid . . .	(—)	—	(—)	(+)	(+)	+	(—)	—	(—)	(—)	—	(+)	+
Pyruvic acid . . .	(—)	(—)	—	+	(+)	+	+	—	(+)	—	—	(+)	+
Levulinic acid . . .	(—)	—	—	—	—	—	—	—	+	—	—	—	
Oxalic acid . . .	—	—	—	—	—	—	—	—	—	—	—	—	
Malonic acid . . .	(—)	—	—	—	—	—	—	—	(—)	—	—	+	+
Ethylmalonic acid . . .	—	—	—	—	—	—	—	—	(—)	—	(+)	—	
Dimethylmalonic acid . . .	—	—	—	—	—	—	—	—	(—)	—	—	—	
Diethylmalonic acid . . .	—	—	—	—	—	—	—	—	—	—	—	—	
Succinic acid . . .	(+)	—	—	+	+	+	+	(—)	+	—	(—)	(+)	+
Methylsuccinic acid . . .	—	—	—	—	—	—	—	—	—	—	—	—	+
Glutaric acid . . .	—	—	—	—	—	(—)	—	—	—	—	—	(+)	+
Adipic acid . . .	—	—	—	—	—	—	—	—	(—)	—	—	—	(—)
Pimelic acid . . .	—	—	—	—	—	—	—	—	(—)	—	—	—	
Suberic acid . . .	—	—	—	—	—	—	—	—	(+)	—	—	—	
Azelaic acid . . .	—	(—)	—	—	—	(—)	—	—	(+)	—	(—)	(—)	
Sebacic acid . . .	—	—	—	—	—	—	—	—	(+)	—	(—)	(—)	
Maleic acid . . .	—	—	—	—	—	—	—	—	—	—	—	—	
Fumaric acid . . .	—	—	—	—	—	(—)	(+)	—	+	—	(—)	+	+
Citraconic acid . . .	—	—	—	—	—	—	—	—	—	—	(—)	+	
Mesaconic acid . . .	—	—	—	—	—	—	—	—	—	—	—	(+)	
Tricarballic acid . . .	—	—	—	—	—	—	—	—	(—)	—	(—)	—	
Aconitic acid . . .	(—)	—	—	—	—	(+)	+	—	(—)	—	(—)	—	
Malic acid . . .	+	—	—	+	+	+	+	(—)	+	(—)	+	+	+

¹ den Dooren de Jong, 1926.

TABLE 18 (continued)

Water with 0.1% K_2HPO_4 , 1% Am_2SO_4 , 1% $CaCO_3$, 0.5% of the undermentioned compounds	<i>Bac. vulgaris</i>	<i>Bac. mycoides</i>	<i>Bac. polymyxa</i>	<i>B. aerogenes</i>	<i>B. coli</i>	<i>B. prodigiosum</i>	<i>B. herbicola</i>	<i>B. vulgare</i>	<i>Myc. phlaxi</i>	<i>Microc. albus</i>	<i>Sarc. lutea</i>	<i>Ps. fluorescens</i>	<i>Ps. aminovorans a</i>	<i>Sp. tenue</i>
tartaric acid	—	—	—	—	—	—	—	+	(—)	—	—	—	+	—
acetic acid	—	—	—	—	—	—	—	—	—	—	—	—	—	—
tric acid	(+)	—	—	+	—	+	+	+	+	—	+	+	+	+
phenylacetic acid	—	—	—	+	—	+	—	—	—	—	—	—	—	—
Phenylpropionic acid	—	—	—	—	—	—	—	—	—	—	—	—	(—)	—
phenylglycollic acid	—	—	—	—	—	—	—	—	(—)	—	(—)	—	(—)	—
mannic acid	—	—	—	—	—	—	—	—	+	—	(—)	—	(—)	—
Cumic acid	—	—	—	—	—	—	—	—	—	—	—	—	—	—
cyclohexanecarbonic acid	—	(—)	—	—	—	—	—	(—)	(—)	—	(—)	—	—	—
nic acid	—	—	—	+	—	—	—	(+)	(+)	—	(—)	+	—	—
nitroacetic acid	—	—	—	—	—	—	—	—	—	—	—	—	—	—
hydroglycollic acid	—	—	—	—	—	—	—	—	—	—	—	—	—	—
malic acid	—	—	—	—	—	—	—	—	(—)	—	(—)	—	—	—
richloracetic acid	—	—	—	—	—	—	—	—	(—)	—	—	—	—	—
romic acid	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Brompropionic acid	—	—	—	—	—	+	—	—	+	—	—	—	—	—
Brompropionic acid	—	(—)	—	—	—	—	—	+	(—)	—	(—)	+	—	—
richlorbutyric acid	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Brombutyric acid	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Bromisobutyric acid	—	—	—	—	—	—	—	—	(—)	—	(—)	—	—	—
omnucic acid	(—)	(—)	—	(+)	(+)	(+)	(+)	—	+	—	(—)	+	—	+
Water with 0.1% K_2HPO_4 , 1% Am_2SO_4 , 1% $CaCO_3$, 1.0% of the undermentioned compounds														
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(+)	(—)	—	+	+	(—)
hyl alcohol	(—)	—	—	(+)	(—)	(+)	—	—	(+)	(—)	—	+	+	(—)
opyl alcohol	(—)	—	—	(—)	(—)	(—)	(—)	(—)	(—)	(—)	—	+	+	(—)
propyl alcohol	—	—	(—)	(—)	—	(—)	(—)	(—)	(+)	(—)	(—)	—	—	(—)
ethyl alcohol	—	—	(—)	—	—	(+)	(—)	(—)	(+)	(—)	(+)	+	+	(+)
nobutyl alcohol	(—)	—	(—)	—	(—)	(—)	(—)	(—)	(+)	(—)	(+)	—	—	(+)
secondary butyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	(—)	(—)	(—)	—	(—)
tertiary butyl alcohol	(—)	—	—	(—)	(—)	(—)	—	—	(—)	(—)	(—)	(—)	—	(—)
nyl alcohol	(—)	—	—	(—)	(—)	(—)	—	—	(+)	—	(—)	+	—	(—)
nyl alcohol	(—)	—	—	(—)	(—)	(—)	—	—	(—)	—	(—)	—	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	—	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	—	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	+	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol	(—)	—	—	—	(—)	(—)	—	—	(—)	—	(—)	(—)	—	(—)
ethyl alcohol . . .														

TABLE 18 (continued)

Tapwater with 0.1% K_2HPO_4 , 0.1% Am_2SO_4 , 1% $CaCO_3$, 1.0% of the undermentioned compounds	<i>Bac. vulgaris</i>	<i>Bac. mycoides</i>	<i>Bac. polymyxa</i>	<i>B. aerogenes</i>	<i>B. coli</i>	<i>B. prodigiosum</i>	<i>B. herbicola</i>	<i>B. vulgare</i>	<i>Myc. phlei</i>	<i>Microc. albus</i>	<i>Sarc. lutea</i>	<i>Ps. fluorescens</i>	<i>Ps. aminovorans</i> α
Styron	—	—	—	—	—	—	—	—	—	—	—	—	
Saligenin	—	—	—	—	—	—	—	—	—	—	—	—	
Cyclohexanol	—	—	—	—	—	—	—	—	—	—	—	—	
1-2-Methylcyclohexanol	—	—	—	—	—	—	—	—	(+)	—	(—)	—	
1-3-Methylcyclohexanol	—	—	—	—	—	—	—	—	(—)	—	(—)	—	
1-4-Methylcyclohexanol	(—)	—	—	—	—	—	—	—	(—)	—	(—)	—	
Ethyleneglycol	—	—	—	—	—	—	—	—	—	—	—	—	
α-Propyleneglycol	(—)	—	—	—	(—)	—	—	—	(—)	—	—	—	
Trimethyleneglycol	(—)	—	—	—	—	—	—	(—)	(—)	—	—	+	
2-3-Butyleneglycol	(—)	—	(—)	+	(—)	(—)	—	—	+	(—)	(—)	—	
Pinakon	—	—	—	—	—	—	—	—	—	—	—	—	
Glycerol	+	(+)	+	+	+	+	+	+	(+)	—	+	+	
Erythritol	—	—	—	—	—	+	—	—	—	—	—	—	
Pentaerythritol	—	—	—	—	—	—	—	—	—	—	—	—	
Adonitol	(—)	—	—	+	(—)	+	(—)	—	—	—	—	—	
Quercitol	—	—	—	—	—	—	—	—	—	—	—	—	
Arabinose	+	(—)	+	+	+	(—)	+	(+)	+	—	(+)	+	
Xylose	+	(—)	+	+	+	(+)	+	(+)	+	(—)	+	+	
Rhamnose	(+)	+	(—)	+	+	+	+	(+)	+	(—)	+	+	
Mannitol	+	(—)	+	+	+	+	+	+	+	(+)	+	+	
Sorbitol	(+)	—	—	+	+	+	—	(—)	(—)	—	(+)	+	
Dulcitol	—	—	—	(—)	—	—	—	—	—	—	—	—	
Inositol	+	(—)	—	+	(+)	+	+	(—)	—	+	—	+	
Glucose	+	(+)	+	+	+	+	+	+	+	(—)	+	+	
Mannose	(+)	(—)	+	+	+	+	+	+	+	(—)	+	+	+
Galactose	(—)	(—)	+	+	+	+	+	(+)	+	(—)	+	+	
Levulose	+	(+)	+	+	+	+	+	+	+	—	(+)	+	
Gluconic acid	(+)	(+)	(+)	(+)	(+)	(+)	(—)	(+)	(—)	(—)	—	(+)	
Saccharic acid	+	—	—	+	+	(+)	(+)	(—)	(+)	—	+	+	
Mucic acid	+	—	—	(—)	(+)	—	(+)	(—)	(—)	—	(+)	(+)	
Tapwater with 0.1% KCl, 1% Mg. Phosphate, to which is added 0.5% of the hydro- chloride of the undermentioned compounds													
Methylamine	(—)	—	—	—	—	—	—	—	—	—	—	—	+
Dimethylamine	(—)	—	—	—	—	—	—	—	—	—	(—)	—	+
Trimethylamine	—	—	—	—	—	—	—	—	(—)	—	—	—	+
Tetramethylamm. hydr.	—	—	—	—	—	—	—	—	(—)	—	—	—	(—)
Ethylamine	—	—	—	—	—	—	—	—	(—)	—	—	—	(+)
Diethylamine	—	—	—	—	—	—	—	—	(—)	—	—	—	(—)
Triethylamine	—	—	—	—	—	—	—	—	(—)	—	—	—	(—)
Tetraethylamm. hydr.	—	—	—	—	—	—	—	—	(—)	—	—	—	(—)
Propylamine	—	—	—	—	—	—	—	—	—	—	—	—	+
Isopropylamine	—	—	—	—	—	—	—	—	—	—	—	—	+
Dipropylamine	—	—	—	—	—	—	—	(—)	—	—	—	—	—

TABLE 18 (continued)

	<i>Bac. vulgaris</i>	<i>Bac. mycoides</i>	<i>Bac. polymyxa</i>	<i>B. aerogenes</i>	<i>B. coli</i>	<i>B. prodigiosum</i>	<i>B. herbicola</i>	<i>B. vulgaris</i>	<i>Myc. phidii</i>	<i>Microc. albus</i>	<i>Sarc. lutea</i>	<i>Ps. fluorescens</i>	<i>Ps. ammoniarius</i> α	<i>Sp. tenuis</i>
2% water with 0.1% KCl, 0.2% protein, to which is added 0.1% of the hydrocarbon of the water-soluble compound is														
propylamine	—	—	—	—	—	—	—	—	(—)	—	—	—	(+)	—
tripropylamm. hydr.	—	—	—	—	—	—	—	—	(—)	—	—	—	(+)	—
ethylamine	—	—	—	—	—	—	—	—	—	—	—	—	(+)	—
butylamine	—	—	—	—	—	—	—	—	—	—	—	—	(+)	—
isobutylamine	—	—	—	—	—	—	—	—	(—)	—	—	—	(—)	—
isobutylamine	—	—	—	—	—	—	—	—	(—)	—	—	—	(+)	—
nylamine	—	—	—	—	—	—	—	—	—	—	—	—	(+)	—
amylamine	—	—	—	—	—	—	—	—	—	—	—	—	(—)	—
amylamine	—	—	—	—	—	—	—	—	(—)	—	—	—	(—)	—
nylamine	—	—	—	—	—	—	—	—	—	—	—	—	(—)	—
ethylamine	—	—	—	—	—	—	—	—	—	—	—	—	(+)	—
ethanolamine	—	—	—	—	—	—	—	—	(+)	—	—	—	(+)	(+)
methanolamine	—	—	—	—	—	—	—	—	(—)	—	(—)	—	(—)	(—)
oline	—	—	—	—	—	—	—	—	(—)	—	(—)	—	(+)	—
ucosamine	(+)	(—)	+	+	(+)	+	+	(—)	(—)	—	+	+	(+)	(—)
urine	—	—	—	—	—	(—)	—	—	(—)	—	—	—	(+)	(—)
ylamine	—	—	—	—	—	—	—	—	(—)	—	—	—	(—)	—
ethylene diamine	(—)	(—)	—	—	—	—	—	—	(—)	—	(—)	—	(—)	—
methylethylene diamine	—	(—)	—	—	—	—	—	+	+	—	—	+	(+)	—
trimethylethylene tetramine	—	—	—	—	—	—	—	—	—	—	—	—	(+)	—
nzylamine	—	—	—	—	—	—	—	—	—	—	—	—	(+)	—
Phenylethylamine	—	—	—	—	—	—	—	—	—	—	—	—	(—)	—
Phenylethylamine	—	—	—	—	—	(+)	—	—	(—)	—	(—)	—	(—)	—
stamine	—	—	—	—	—	—	—	—	(—)	—	(—)	—	(—)	—
peridine	—	—	—	—	—	—	—	—	(—)	—	(—)	—	(—)	—
perazine	—	—	—	—	—	—	—	—	(—)	—	(—)	—	(—)	—
rol	—	—	—	—	—	—	—	—	(—)	—	(—)	—	(—)	—
ridine	—	—	—	—	—	—	—	—	(—)	—	(—)	—	(—)	—
vdrazine	—	—	—	—	—	—	—	—	(—)	—	(—)	—	(—)	—
2% water with 0.1% K ₂ HPO ₄ , glucose, 1% CaCO ₃ , to which is added 0.1% of the under-mentioned compounds as hydrochloride or chloride														
ethylamine	(—)	(—)	(—)	+	(+)	(+)	+	(+)	+	—	(+)	(+)	(+)	—
methylethylamine	(—)	(—)	(—)	+	(—)	(—)	+	(—)	(+)	—	(—)	(—)	(+)	—
trimethylethylamine	(—)	(—)	(+)	+	(—)	(—)	+	(—)	(+)	—	(—)	(—)	(—)	—
tetramethylethylamm. hydr.	(—)	(—)	+	+	(+)	(+)	+	(+)	(+)	—	(—)	(—)	(+)	—
ethylamine	—	—	+	+	(—)	(+)	+	(—)	(+)	—	(—)	(—)	(—)	—
diethylamine	—	—	+	+	(—)	(—)	+	(—)	(—)	—	(—)	(—)	(—)	—
triethylamine	—	—	+	+	(—)	(—)	+	(—)	(—)	—	(—)	(—)	(—)	—
tetraethylamm. hydr.	(—)	(—)	+	+	(—)	(+)	+	(—)	(+)	—	(—)	(—)	(—)	—
propylamine	(—)	—	+	+	(—)	(—)	+	(—)	(+)	—	(—)	(—)	(—)	—
isopropylamine	(—)	—	+	+	(—)	(+)	+	(—)	(+)	—	(—)	(—)	(—)	—
tripropylamine	—	—	+	+	(—)	(—)	+	(—)	(+)	—	(—)	(—)	(—)	—
tetrapropylamm. hydr.	—	—	+	+	(—)	(—)	+	(—)	(+)	—	(—)	(—)	(—)	—

TABLE 18 (continued)

	<i>Bac. subtilis</i>	<i>Bac. mycoides</i>	<i>Bac. polymyxa</i>	<i>B. aerogenes</i>	<i>B. coli</i>	<i>B. prodigiosum</i>	<i>B. herbaria</i>	<i>B. vulgare</i>	<i>Myc. piliferi</i>	<i>Microc. albus</i>	<i>Sarc. lutea</i>	<i>Ps. fluorescens</i>	<i>Ps. aminovorans</i> α	<i>Sp. tenue</i>
water with 0.1% K_2HPO_4 , lysine is added 1% of the total dissolved compounds														
lysine	(+)	—	—	(+)	—	+	—	+	(—)	—	(+)	(+)	—	—
hydrochloride	(—)	(—)	(—)	(—)	(+)	+	—	—	(+)	(—)	(—)	(+)	—	—
tryptophan	—	—	—	—	—	(—)	—	(—)	(—)	—	—	—	—	—
Aminobutyric acid	—	—	—	(—)	—	(—)	—	(—)	(—)	—	(—)	(—)	—	—
Aminoisobutyric acid	—	—	—	—	—	—	—	(—)	(—)	—	(—)	(—)	—	—
Methylbutyric acid	—	(—)	—	—	—	—	(—)	(+)	(+)	—	+	+	—	—
Aminovaleric acid	(—)	(—)	—	—	—	(—)	—	+	+	—	(+)	+	+	(+)
Aminovaleric acid	—	—	—	(—)	+	(—)	—	+	+	—	(+)	+	+	(+)
Aminoisovaleric acid	—	—	—	—	—	—	—	(+)	(—)	—	(+)	+	+	(—)
Aminocaproic acid	(—)	(—)	—	—	—	(—)	—	(—)	+	—	+	(—)	+	(—)
cysteine	(—)	(—)	(—)	(—)	—	(+)	—	(+)	+	—	+	+	+	(—)
glutamine	(—)	(—)	—	—	—	(—)	—	(—)	(+)	—	(—)	+	+	(—)
Aminocaprylic acid	—	—	—	—	—	—	—	—	(—)	—	(—)	—	—	—
sparteic acid	(+)	(—)	—	+	(+)	+	+	+	(+)	—	+	+	+	(+)
lutamic acid	(+)	—	—	+	+	+	+	+	+	(+)	+	+	+	+
water with 0.1% K_2HPO_4 , lysine and 1% Cat O_2 , to which is added 1% of the total dissolved compounds														
glycocol	(+)	+	+	+	+	+	+	+	+	—	+	+	—	—
treosine	(—)	—	+	+	(—)	(+)	+	+	+	—	(—)	+	—	—
lysinehydrochloride	(+)	—	+	+	(—)	(—)	+	(+)	+	—	(—)	+	(+)	(+)
acetylglucosyl	+	(—)	+	+	(+)	+	+	(+)	(+)	(—)	(+)	(—)	(—)	(—)
benzylglycocol	(—)	(—)	(—)	(+)	(—)	(—)	+	(—)	(+)	—	(—)	(—)	(—)	(—)
benzylglycine- α - carbonic acid	—	—	+	+	(+)	(+)	+	(—)	(+)	(—)	(+)	(+)	(+)	(+)
pyruvic acid	—	—	+	+	(+)	(+)	+	+	+	—	+	(+)	(+)	(—)
benzylaminoacetic acid	(+)	—	+	+	(—)	(—)	+	(+)	+	—	(+)	(—)	—	(—)
aurine	(—)	(—)	(+)	+	(—)	—	+	+	+	—	+	+	—	—
Alanine	+	(+)	+	+	+	+	+	+	+	(—)	+	+	(+)	(+)
Alanine	(+)	(+)	+	+	(+)	+	+	+	+	—	(—)	(+)	(+)	(+)
benzylalanine	(+)	(—)	(+)	+	+	+	+	(+)	+	—	+	+	(+)	(+)
tyrosine	(+)	(—)	(+)	+	+	+	+	+	+	—	+	+	(+)	(+)
benzylalanine	(+)	(—)	(+)	+	(—)	(—)	+	—	+	—	(—)	(—)	(—)	(—)
lysine	(+)	—	(—)	+	+	+	+	(—)	+	—	(—)	(—)	(—)	(—)
lysine hydrochloride	+	+	+	+	+	+	+	+	+	(+)	+	+	(+)	(+)
tryptophan	(—)	—	+	+	+	+	+	+	+	—	(—)	+	—	—
Aminobutyric acid	(—)	—	+	(+)	+	+	+	(+)	+	—	(—)	+	—	—
Aminobutyric acid	(—)	(—)	(—)	+	(—)	(—)	+	(—)	+	—	(—)	(—)	—	—
Amino- α -methyl butyric acid	—	—	+	+	(+)	(+)	+	(+)	(—)	—	(—)	(—)	—	—
Aminovaleric acid	(+)	—	+	+	+	(+)	+	(+)	(—)	—	+	+	+	+
Aminovaleric acid	(+)	(+)	(+)	+	+	(+)	+	(—)	+	—	—	+	+	+
Aminoisovaleric acid	+	(—)	+	+	+	(+)	+	+	+	—	(+)	(+)	+	+
Aminocaproic acid	(—)	—	+	+	+	+	+	(+)	+	(—)	(+)	+	+	+
cysteine	+	+	+	+	+	(+)	+	+	+	—	+	+	—	—
glutamine	+	(—)	+	+	+	+	+	+	+	—	+	+	—	—
Aminocaprylic acid	(—)	—	+	+	(+)	(+)	+	(+)	(—)	—	(—)	(+)	—	—
sparteic acid	+	(—)	+	+	+	+	+	(+)	+	—	+	+	+	+
lutamic acid	+	+	(+)	+	+	+	+	(+)	+	(—)	+	+	+	+

TABLE 18 (continued)

Tapwater with 0.1% K_2HPO_4 , to which is added 0.5% of the undermentioned compounds	<i>Bac. vulgatus</i>	<i>Bac. mycoides</i>	<i>Bac. polymyxa</i>	<i>B. aerogenes</i>	<i>B. coli</i>	<i>B. prodigiosum</i>	<i>B. herbicola</i>	<i>B. vulgare</i>	<i>Myc. phlei</i>	<i>Microc. albus</i>	<i>Sarc. lutea</i>	<i>Ps. fluorescens</i>	<i>Ps. aminovorans</i> a
Formamide	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	-
Acetamide	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	+
Propionamide	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	+
Butyramide	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	+
Isobutyramide	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	+
Valeramide	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	+
Capronamide	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	+
Palmitamide	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	+
Stearamide	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	+
Lactamide	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	+
Oxamide	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	+
Methyloxamide	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	+
Malonamide	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	+
Succinamide	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	+
Fumaramide	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	+
Succinimide	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	+
Glutarimide	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	+
Asparagine	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	+
Thio-acetamide	(-)	(-)	-	(+)	(+)	(+)	(-)	(+)	(+)	-	(+)	(+)	+
Tapwater with 0.1% K_2HPO_4 , 1% glucose, 1% $CaCO_3$, to which is added 0.1% of the under- mentioned compounds													
Formamide	+	(-)	+	(+)	+	+	+	-	-	(-)	(-)	+	
Acetamide	(-)	(+)	(-)	(+)	+	(-)	+	-	-	(-)	(-)	(-)	
Propionamide	(+)	(-)	+	(+)	+	(-)	+	-	+	(-)	(+)	(-)	
Butyramide	(+)	(-)	+	(+)	(-)	(-)	+	-	+	(-)	(+)	(-)	
Isobutyramide	(-)	(-)	(+)	(+)	(-)	(+)	+	-	+	+	(+)	(+)	
Valeramide	(+)	(-)	+	(-)	(-)	(-)	+	-	+	+	(+)	(-)	
Capronamide	(+)	(-)	(+)	(-)	(-)	(-)	+	-	(+)	(-)	(+)	(-)	
Palmitamide	(-)	(-)	-	(-)	(-)	(-)	+	-	(-)	(-)	(+)	(-)	
Stearamide	(-)	(-)	-	(-)	(-)	(-)	+	-	(-)	(-)	(+)	(-)	
Lactamide	(-)	(-)	+	+	(-)	+	+	-	+	+	(+)	(+)	
Oxamide	(+)	(-)	+	(-)	(-)	(-)	+	(-)	(+)	(-)	(-)	(-)	
Methyloxamide	(-)	(-)	+	(+)	(+)	(-)	+	(-)	(+)	(-)	(-)	(-)	
Malonamide	(-)	(-)	+	(-)	(-)	(-)	+	(-)	(+)	(-)	(-)	(-)	
Succinamide	(+)	(-)	(+)	+	(-)	+	+	(+)	(+)	(-)	(-)	(+)	
Fumaramide	(-)	(-)	(-)	+	(-)	(-)	+	(-)	(+)	(-)	(+)	(+)	
Succinimide	+	(-)	+	+	(-)	(+)	+	(+)	(+)	(-)	(-)	(+)	
Glutarimide	(-)	(-)	(+)	+	(-)	(+)	+	(+)	(+)	(-)	(-)	(+)	
Asparagine	+	(+)	+	+	+	+	+	(+)	+	+	(-)	(+)	
Thio-acetamide	(-)	(-)	+	+	(+)	(+)	+	(+)	+	(-)	(-)	(+)	

TABLE 18 (continued)

TABLE 18 (*continued*)

Tapwater with 0.1% K_2HPO_4 , 1% glucose, 1% C_4O_3 , to which is added 0.1% of the under- mentioned compounds	<i>Bac. vulgaris</i>	<i>Bac. mycoides</i>	<i>Bac. polymyxa</i>	<i>B. aerogenes</i>	<i>B. coli</i>	<i>B. prodigiosum</i>	<i>B. hericola</i>	<i>B. vulgare</i>	<i>Myc. phlei</i>	<i>Microc. albus</i>	<i>Sarc. lutea</i>	<i>Ps. fluorescens</i>	<i>Ps. ammoniacus</i> α
Creatine	(—)	—	+	+	(—)	+	+	(—)	(+)	—	(—)	(—)	
Creatinine	(—)	(—)	+	+	(—)	+	+	(+)	(—)	—	(+)	+	
Arginine	+	+	+	+	+	+	+	+	+	—	+	+	
Parabanic acid	+	—	+	+	+	(+)	+	+	+	(+)	+	+	
Alloxan	(+)	(—)	+	+	+	+	+	(+)	+	(—)	+	+	
Alloxantine	+	(—)	+	+	+	+	+	(—)	+	—	+	+	
Barbituric acid	(—)	(—)	+	+	(—)	+	+	(+)	(+)	—	(+)	(+)	
Thiobarbituric acid	+	—	(+)	+	(—)	(+)	+	(—)	(—)	—	(—)	(—)	
Violuric acid	—	—	—	—	(—)	+	—	—	—	—	—	—	
Allantoin	+	(—)	+	+	(+)	+	+	(—)	+	(+)	+	(—)	
Guanine	(+)	(—)	(+)	+	(+)	(+)	+	(—)	(+)	—	+	(—)	
Xanthine	+	(—)	+	+	+	+	+	+	(+)	(—)	(+)	+	
Uric acid	(+)	(—)	(+)	+	(+)	+	+	(+)	(+)	—	(+)	(+)	
Theobromine	—	—	+	+	(+)	(+)	+	(+)	(+)	—	(—)	(—)	
Theophylline	—	—	(—)	(+)	(—)	(—)	(+)	(—)	(+)	—	(—)	(—)	
Caffeine	—	—	(+)	+	(—)	(—)	+	(+)	(—)	—	(—)	(—)	

[Continued from p. 183]

growth are formic acid, glycine and ethyl alcohol in the case of *Bact. typhosum*, though the last two can function in the case of several organisms studied by den Dooren de Jong.

A factor contributing to the value of a compound for growth is the degree of dilution at which it is operative, which in its turn depends on the affinity of the substance for its enzyme. The high affinity of glucose in comparison with other carbohydrates is shown in Fig. 18,¹ whilst Friedlein² has determined the minimum concentration of certain compounds at which growth can occur (Table 19). The figures there given indicate degrees of growth maintained on the same medium through several generations; figures in brackets indicate growth not subcultivable in the same medium. The minimum concentration of ammonium required was determined on a synthetic medium with excess (1%) sodium lactate.

Specific nutritional requirements

The use of complex media dates from the days of medical bacteriology when pathogenic organisms were isolated with the

¹ Fujita & Kodama, *Bioch. Z.*, **271**, 186, 1934.

² Friedlein, 1928.

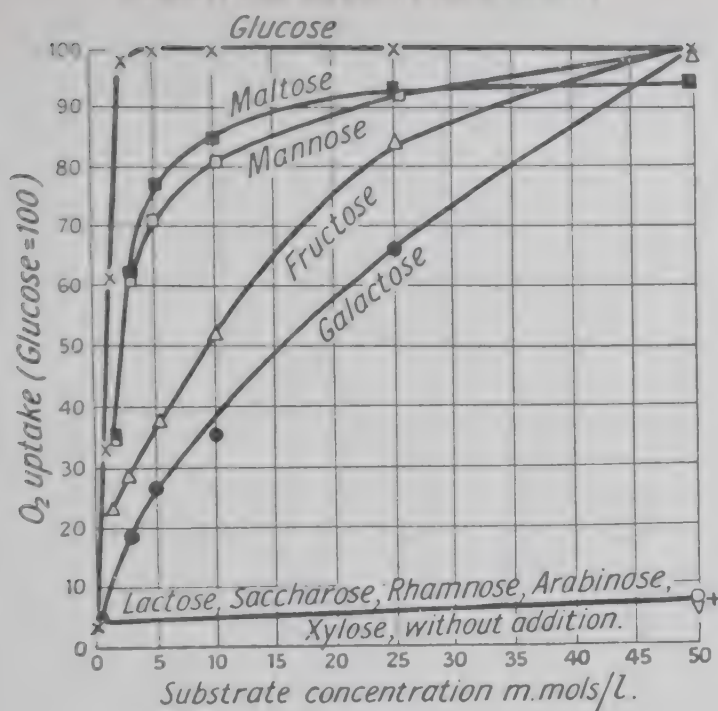


FIG. 18

TABLE 19¹

Organism	Growth on varying concentrations of sodium lactate			
	0.001%	0.01%	0.1%	1.0%
<i>B. paratyphosus B</i>	0	(1)	3	4
<i>B. coli</i>	0	(1)	4	4
Organism	Growth on varying concentrations of glucose			
	0.00016%	0.0016%	0.016%	0.16%
<i>B. paratyphosus B</i>	0	(1)	3-2	3
<i>B. coli</i>	0	0	2	3
<i>B. pyocyaneus</i>	0	0	2	4
Organism	Growth on varying concentrations of sodium succinate			
	0.00144%	0.0144%	0.144%	1.44%
<i>B. paratyphosus B</i>	0	(1)-(2)	3-4	4
Organism	Growth on varying concentrations of ammonium chloride			
	0.0001%	0.001%	0.01%	0.1%
<i>B. paratyphosus B</i>	(2)*	(2)*	3	4
<i>B. coli</i>	(1)*	(1)*	4	4

* Growth which is not subcultivable in the same medium is obtained in the absence of any ammonium chloride.

¹ Friedlein, 1928.

object of establishing their relationship to disease. The prime object was to cultivate the organism, keep it in cultivation and maintain its pathogenicity, its actual growth requirements being a matter of little concern. It fairly early, however, became apparent that many saprophytes and some pathogens could be cultivated on simple synthetic media with nitrogen supplied as ammonium salts and carbon as sugars, alcohols or organic acids. The first serious attack on the problem of bacterial growth requirements was made by Fildes,¹ who put forward the hypothesis—now a well-established theory—that parasitism is caused by loss of enzymes necessary to synthesise cell material. Thus the first stage in parasitism occurs when an organism uses as a food material a compound which occurs naturally only as the result of the activity of some other living system; the change of autotrophant to heterotrophant corresponds to such a step. A second stage is reached when the cell is no longer able to live on simple synthetic media as defined above, but requires as well at least one other compound supplied by the activity of another organism for use as a structural unit or for metabolic processes. *E. coli* (most strains) exemplifies stage 1 and *Proteus vulgaris*—which can develop on simple synthetic media plus nicotinic acid—is the classical case of stage 2. From this point there can be constructed a series of organisms with steadily increasing nutritional requirements—or “exactingness”—which may include any or all of the B group of vitamins, many amino-acids and some purines and pyrimidines. The view that nutritional exactingness is correlated with enzymic deficiency now admits of no doubt; this seems to occur by the continued growth of an organism in surroundings where nutritional units occur in great quantity; if in such surroundings a mutant arises which has lost an enzyme necessary for the production of an essential compound, it is then able to supply its need from its surroundings and survive. What causes such a mutant to outgrow the parent strain is not clear. Thus many organisms unable to synthesise riboflavin occur in milk, those requiring hæmatin in blood and tissues, and so on.

Study of the vitamin requirements of bacteria has disclosed many facts of great biological and biochemical importance. Every member of the B or water-soluble group of vitamins has now been found necessary for some nutritionally exacting organism or other; several were first discovered as growth factors for bacteria, their importance in animal and plant nutrition becoming apparent later. Furthermore, in every case where an organism dispenses with any member of the B group in its food supply it can be shown to synthesise it, heated extracts of such organisms supplying the

¹ Fildes, 1934.

specific nutritional requirements of exacting strains. This, as has been emphasised by Knight, shows an underlying unity of metabolic needs throughout the animal and microbic world, extending in many instances to the plant kingdom also. It has also led to the use of micro-organisms in the study of the function of vitamins in metabolism. This was first demonstrated by Lwoff and Lwoff, using a method which has now become general. The exacting organism (in this instance *H. parainfluenzæ*) was grown in a sub-optimal concentration of the growth factor (coenzyme I) and the activity of its enzyme systems in washed suspension compared with that of the same organism grown in optimal amounts of the same growth factor. In this case the suspensions of the deficient organism were found to be suboptimal in respect of dehydrogenases for glucose, hexosemonophosphate, pyruvate, fumarate, ethanol, asparagine and *d*- and *l*-valine, but gave increased response approaching normal for all these substrates when incubated for a short period with coenzyme I.¹ The applications of this method are manifold and special examples will be considered later.

In addition to cases in which an organism is totally unable to synthesise a growth factor, cases are known when it cannot do so at the optimal rate, so that growth rate is limited by the rate of synthesis of the vitamin and can be increased by its supply in the medium. In many cases where organisms synthesise their own growth factors they do so at a rate in excess of their needs and the excess passes out and can be used by deficient organisms. Cases of symbiosis are attributable to this cause.

This excessive rate of synthesis of B vitamins is responsible for the phenomenon of refection in animals kept on a synthetic diet. Here growth which has ceased owing to dietary deficiency of some water-soluble factor is found to be spontaneously resumed due to the contribution of the gut flora to the vitamin supply. Many insects also are unable to supply their own B vitamins when raised in sterile conditions on a synthetic diet but can be reared successfully on the same diet if allowed to develop a normal bacterial flora.

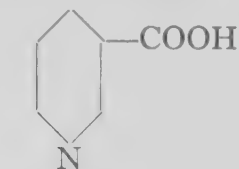
The study of the specific nutritional requirements of micro-organisms sheds light on the chemical stages by which they are synthesised in the cell. In general a slight change in chemical configuration renders the compound useless to the cell; in cases where a change is tolerated the growth induced is usually slower. On the other hand some part of the molecule, e.g. aneurin in place of aneurinphosphate, may serve the purpose of the cell, indicating that the loss of synthetic powers in respect of the compound is not complete; such instances will be dealt with under the more detailed consideration of different factors.

¹ Lwoff & Lwoff, 1937.

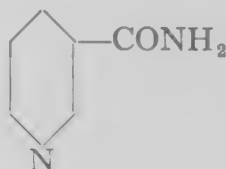
Yet another phenomenon to be considered is the regaining of lost synthetic powers. This can occasionally be achieved by the subcultivation of large amounts of the organism in decreasing concentrations of the necessary compound. Several examples of this are known, though the mechanism of the process is at present uncertain. Perhaps the enzyme in question is not completely absent throughout the entire population but is present in such small amount that the culture generally dies out before enough molecules are synthesised to serve the needs of the cell. The technique employed would serve to foster those members of the population able to synthesise the factor rather more rapidly until a stage was reached when a cell population was formed able to synthesise the compound at a rate compatible with survival.

Some growth factors will now be considered in detail.

Nicotinic acid and amide



nicotinic acid



nicotinamide

Nicotinic acid is one of the commonest growth factors needed by bacteria; in other words, the ability to synthesise it is one most frequently lost. This may be due to its very wide distribution in soil and natural media. It forms the sole factor required by *Proteus vulgaris* and one recorded strain of *coli* and enters into the requirements of a large number of highly exacting organisms. Its absence in animal nutrition causes blacktongue in the dog and pellagra in the pig and in man; it is also necessary for the growth of isolated roots in plants and for some yeasts.

One function of nicotinic acid is to supply the nicotinamide fraction of coenzymes I and II; hence all organisms requiring nicotinic acid can replace it by nicotinamide, but the converse is not true.

The specificity of nicotinic acid and amide in the growth of bacteria is fairly strict. Generally speaking, substitution in the side chain is tolerated, methyl ethyl and propyl esters and nicotinyl glycine all giving some activity with the organisms tested, but the introduction of new substituents in the ring, e.g. the change of the —COOH to the 2- or 4-position, renders the molecule inactive.¹

In general the function of nicotinic acid and amide seems to be

¹ Knight, 1945.

to provide prefabricated units for the synthesis of coenzymes I and II, which will now be considered.

Adenine pyridine di- and trinucleotides (coenzymes I and II)

The structure of these compounds is given on p. 18, and their relation to nicotinic acid and amide is obvious. Coenzyme I is a constant component of bacteria, boiled washed suspensions of all organisms so far tried restoring the activity of any enzyme system deficient in, but requiring, cozymase.

The *Hamophilus* group of bacteria has long been known to have special growth requirements. It was early shown by Pfeiffer¹ that the organism then known as *B. influenzae*, isolated from the sputum of influenza patients, does not grow on broth unless sterile blood is added. Later it was shown that when cultivated on blood agar plates much larger colonies are formed in the neighbourhood of colonies of other organisms,² and subsequently that these so-called "satellite" colonies occur also round sterile slices of animal or vegetable tissue introduced into the agar.³ This led to the recognition of two factors, both present in blood, necessary to the growth of this organism in broth :^{4, 5} the X factor, which is heat-stable and will be dealt with later, and the V factor, which is heat-labile in alkaline but not in acid solutions.

The V factor was studied by Lwoff and Lwoff⁶ and shown to be identical with coenzyme I prepared from yeast. As mentioned earlier, these workers introduced a method, since of wide application, for using micro-organisms to determine the biochemical action of growth factors. *H. parainfluenzae* was grown in about one-sixth of the amount of coenzyme I needed for optimal growth ; the resulting crop was known as V- cells, and organisms grown in excess of coenzyme I as V+ cells. It was found that washed suspensions of V- cells oxidised those compounds whose oxidation depends on coenzyme I only at a very slow or negligible rate ; when the cells were incubated with coenzyme I the rate of oxidation was increased to that of V+ cells. Evidence was obtained of the mutual interconversion within the cells of coenzymes I and II.

Various derivatives and components of coenzyme I have been tested as growth factors, two strains of *H. parainfluenzae* and four strains of *H. influenzae* giving identical results. The compounds tested were coenzyme I and its reduction product dihydro-coenzyme I ; these gave identical results, which is consistent with their rapid changeover in the cell. Desaminocoenzyme (formed by replacement of $-\text{NH}_2$ by $-\text{OH}$ in position 6 of adenine) was

¹ Pfeiffer, 1893.

² Ghon & Preyss, 1902, 1904.

³ Davis, 1917.

⁴ Triggott & Avery, 1921 (1), (2).

⁵ Fildes, 1921.

⁶ Lwoff & Lwoff, 1937 (1), (2).

nearly as efficient as coenzyme, whilst nicotinamideriboside and coenzyme II both supported a slower growth. These organisms are evidently able to dispense with the adenine portion of the molecule altogether provided that the nicotinamide-ribose link is made, growth in this case being slower. The poor response obtained by coenzyme II suggests that this compound is broken down and rebuilt before use. Nicotinamide cannot be used by this group.

The biosynthesis of coenzyme I therefore occurs in at least four demonstrable steps:

$x \xrightarrow{1} \text{nicotinic acid} \xrightarrow{2} \text{nicotinamide} \xrightarrow{3} \text{nicotinamide nucleoside} \xrightarrow{4} \text{coenzyme I.}$

P. vulgaris fails at 1, but effects 2, 3 and 4. *Pasteurella* strains fail at 1 and 2, but effect 3 and 4. The *Hæmophilus* group fails at 1, 2 and 3 but effects 4.

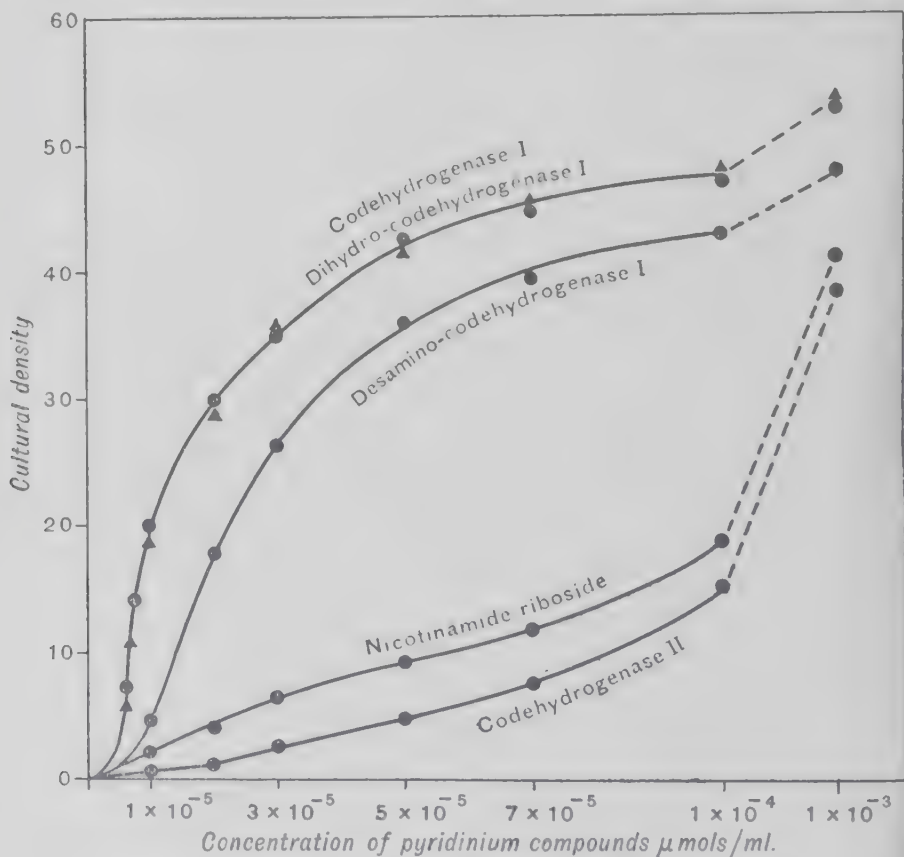


FIG. 19.—Growth of *H. parainfluenzæ* in presence of coenzyme I and some derivatives¹

¹ Gingrich & Schlenk, *J. Bact.*, 47, 543 (1944).

There is, however, evidence indicating that the formation of coenzyme I may not be the only function of nicotinamide in the cell. It has been found that with *Shigella paradysenteriae* the effectiveness as growth factors is in the following order : nicotinic amide, nicotinic acid, coenzyme I, coenzyme II ; after hydrolysis the last two become as effective as nicotinamide.¹

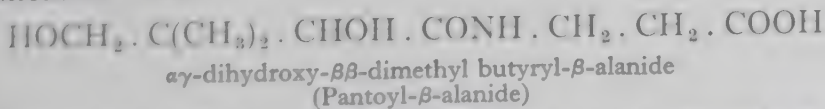
When grown on suboptimal concentrations of nicotinamide the deficient cells display low respiratory activity on glucose ; this is raised by the addition of the four compounds under discussion, their effectiveness for this purpose being in the same order as for growth. As these differences are extremely marked they suggest a function of nicotinamide in respiration apart from the formation of coenzyme I.

Hæmatin as growth factor

It has been mentioned (p. 197) that the *Hæmophilus* group requires a heat-stable X factor usually supplied by sterile blood. It can also be supplied by hæmoglobin 1/10⁶ and by hæmatin, but not by iron-free pyrrol compounds. Conflicting evidence has correlated it with the presence of peroxidase and catalase^{2, 3} ; the fact that these enzymes are both hæmatin compounds probably explains this.

There has been a strong tendency to associate the X factor with respiration. *H. influenzae* and organisms requiring X were usually regarded as strict aerobes, but it was later shown that a number of strains can be grown anaerobically through successive generations and that in these conditions X can be dispensed with.^{4, 5, 6} Substances supplying the X factor in general give a peroxidase reaction with benzidine, as do most bacteria ; this is probably due to their hæmatin content. Organisms requiring the X factor do not give this reaction, nor do the *Clostridia*.⁷ It appears then that X supplies some hæmatin compound (or its precursor) necessary for aerobic oxidations, but its exact nature and function in the cell are still uncertain.

Pantothenic acid



Pantothenic acid functions as a growth factor for animals where its deficiency leads to cessation of growth and greying of hair (rats) and a specific dermatitis in chicks. An unknown factor was first

¹ Saunders *et al.*, 1941.

² Fildes, 1921.

³ Olsen, 1920, 1921.

⁴ Kopp, 1927.

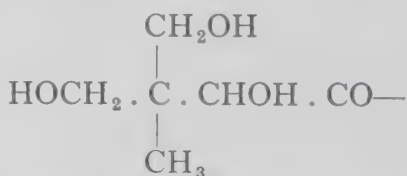
⁵ Baudisch, 1932.

⁶ Anderson, 1931.

⁷ Callow, 1926.

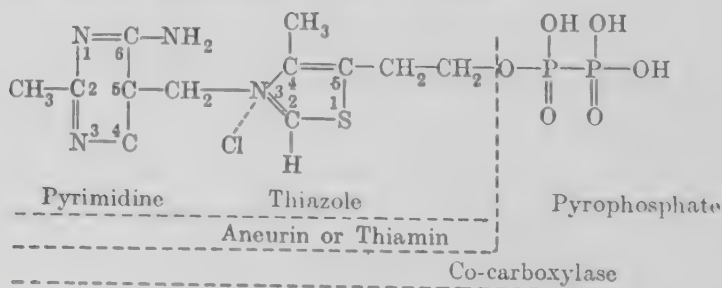
noted in the growth requirements of a strain of yeast by Williams and his colleagues¹ in 1933, and its chemical structure worked out in 1939² and identified with the chick antidermatitis factor.³ The importance of pantothenic acid in microbial and animal physiology was thus discovered almost simultaneously.

Pantothenic acid is an essential growth factor for a large number of bacterial species;⁴ some of these can replace it by β -alanine, fewer by pantoic acid⁵ (or lactone). The synthetic powers of organisms with respect to pantothenic acid and its two hydrolytic products are most apparent among the group of *C. diphtheriae*. Most strains grow on a complicated synthetic medium containing salts, lactate and 20 amino-acids, with pimelic and nicotinic acids and β -alanine as growth factors. Certain *gravis* strains, however, fail on this medium but grow when pantothenic acid is added,⁶ though when the two portions of the molecule are added as the hydrolytic products of pantothenic acid the organism is unable to effect the synthesis. The specificity of both parts of the pantothenic molecule is extremely strict, the only substitute tried giving more than a fraction of 1% of the activity of the natural vitamin being hydroxypantothenic acid



which shows an activity varying between 1% and 25% of that of pantothenic acid with *S. cerevisiae* and some lactic bacteria.⁷

Aneurin (thiamin) vitamin B₁ and co-carboxylase



Thiamin is an essential growth factor for a number of bacteria, including *Staph. aureus*, some propionic bacteria and *S. salivarius*; it is added to the growth media of many lactic organisms

¹ Williams *et al.*, 1933.

² Ibid., 1939. ³ Jukes, 1939. ⁴ Knight, 1945.

⁵ Wooley, 1939.

⁶ Evans *et al.*, 1939.

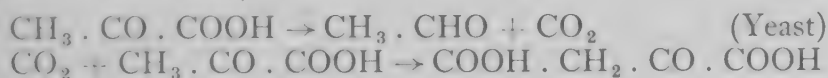
⁷ Mitchell *et al.*, 1940.

which appear not to synthesise it sufficiently rapidly for optimum growth.

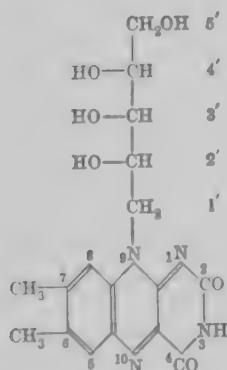
The specificity of thiamin has been tested for in the growth of *Staph. aureus*.^{1, 2} This organism can use an equimolecular mixture of pyrimidine and thiazole as readily as thiamine itself. In this case the CH_3 at 2 and the NH_2 at 6 of pyrimidine are essential, and various substituents at 5 are tolerated, e.g. $-\text{CH}_2 \cdot \text{NH}_2$ and $-\text{CH}_2\text{OH}$ but not $-\text{CH}_3$, indicating that the pyrimidine and thiazole portions are united before use.

In the thiazole ring substitution of the β -hydroxyethyl group at 5 by a γ -hydroxypropyl or a β -hydroxypropyl results in decreased activity. Substitution of methyl for H in position 2 results in lowered activity (35%), whilst $-\text{NH}_2$ in the same position gives complete inactivity. For further details on the substitutions in these compounds the original papers and Knight's monograph should be consulted.

It is certain that the principal function of aneurin is for the synthesis of cocarboxylase. This coenzyme participates in some essential reactions given below and its absence has a corresponding effect on metabolism.



Riboflavin (vitamin B_2)



Riboflavin is an essential growth factor for many bacteria, including haemolytic streptococci, *Str. faecalis*, and for some lactic and propionic bacteria.³ Its importance in the bacterial nutrition was first shown by Orla-Jensen and his co-workers⁴ for some lactic organisms by treating milk with an absorbent charcoal and showing that the treated milk required the addition (among other things) of riboflavin for satisfactory growth.

Later workers,⁵ destroying riboflavin by illumination at pH 10,

¹ Knight, 1937.

² Knight & McIlwain, 1938.

³ Wood *et al.*, 1932.

⁴ Orla-Jensen *et al.*, 1936.

⁵ Snell & Strong, 1939.

showed that out of eleven strains of lactic organisms examined seven dispensed with riboflavin and four (*L. delbrückii*, *L. gangori*, *B. lactis acidii* and *L. casei*) required it; of the eleven not requiring it four were found on examination to synthesise it, and this is probably true for the rest. Many organisms for which riboflavin is not essential grow more rapidly in its presence.

The specificity of riboflavin as a bacterial growth factor is fairly strict. Some interference with the side chain in positions 6 and 7 is tolerated but results in decreased activity; removal of the ribityl group giving lumiflavin or its replacement by *d*- or *l*-arabityl results in complete loss of activity. This is shown in Fig. 20.¹

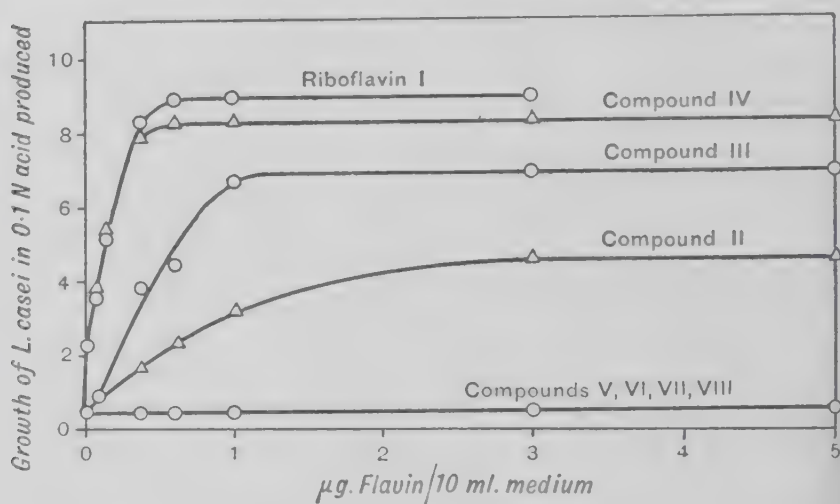


FIG. 20.—Availability of various *d*-riboflavins for growth of *L. casei*²

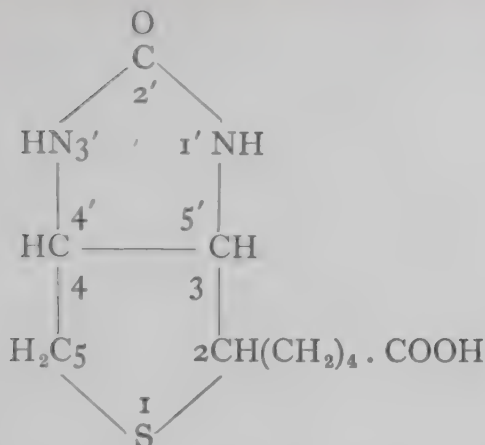
- I. 6, 7-Dimethyl-9-(*d*-1'-ribityl) isoalloxazine (Riboflavin)
- II. 6-Methyl-9-(*d*-1'-ribityl) isoalloxazine
- III. 7-Methyl-9-(*d*-1'-ribityl) isoalloxazine
- IV. 6-Ethyl-7-Methyl-9-(*d*-1'-ribityl) isoalloxazine
- V. 6, 7-Dimethyl-9-(*d*-1'-arabityl) isoalloxazine
- VI. 6, 7-Dimethyl-9-(*l*-1'-arabityl) isoalloxazine
- VII. 6, 7, 9-Trimethyl-9-(*l*-1'-arabityl) isoalloxazine (Lumiflavin)
- VIII. 6, 7-Dimethyl alloxazine (Lumichrome)

V, VI, VII and VIII inactive.

Riboflavin forms part of the prosthetic group of a number of enzymes already known and probably of others still undiscovered (see Chapter I), and it is certainly to this circumstance that its importance as a metabolite is due. Many organisms not requiring it have been shown to synthesise it, but whether it is an essential constituent of all bacterial cells is not known.

¹ Snell & Strong, 1939.

² Ibid., *Enzymol.*, 6, 190 (1939).

Biotin

Biotin is a vitamin which was first detected by studies in yeast nutrition. During investigations into the nature of "bios" Eastcott¹ first separated malt wort into a baryta-soluble and a baryta-insoluble fraction; the latter was again divided into two fractions by adsorption on charcoal. The adsorbed fraction contained a growth factor which was eventually isolated from egg yolk by Kögl and Töhnis,² who used the growth rate of a deficient strain of yeast as the biological test by which to follow the activity of the fractions. From 250 kg. of dried egg yolk they eventually isolated 1.1 mg. (corresponding to a yield of 1.8%) of a crystalline substance, which they called "biotin," containing nitrogen and sulphur; this was actually biotin methyl ester. They showed that this substance is fully active at 0.000025 µg./ml., the lowest concentration then recorded at which a substance is physiologically active. Biotin was soon found to be active in bacterial nutrition and was also identified with coenzyme R, the then unidentified growth factor for *Rhizobia*.^{3, 4} Identification of biotin with an unknown factor in animal nutrition adsorbed on to egg white and hence known as the "egg-white factor" followed. The chemical structure of biotin was established in 1942.^{5, 6}

The extremely low concentration in which biotin functions biologically renders it difficult to devise synthetic media which are inactive in respect of it, glucose and sucrose, unless very specially purified, being liable to contain it.

Many organisms are now known for which biotin (or its methyl ester) is a necessity and others for which it acts as a growth stimulant. Organisms able to grow without biotin have been shown to synthesise it.

¹ Eastcott, 1928. ² Kögl & Töhnis, 1936.

³ Allison & Hoover, 1934.

⁴ West & Wilson, 1939.

⁵ du Vigneaud *et al.*, 1942.

⁶ Hofmann, 1943.

Some interesting facts about the biological synthesis of biotin have been discovered by the joint efforts of organic and biochemists.

It was shown in 1937 by Mueller¹ that certain strains of *C. diphtheriae* require a growth factor contained in Liebig's extract and in many tissue extracts, especially liver. It was finally isolated from cow urine, 455 l. yielding a butanol extract from which was obtained 120 g. of methyl esters from which 0.6 g. of pure pimelic acid was finally prepared ($\text{COOH}(\text{CH}_2)_5\text{COOH}$). Actually this growth factor was only required by two strains of *C. diphtheriae*, Allen and Park 8, and was replaceable by no other dicarboxylic acid of the series. When the structure of biotin became known, it was suggested that pimelic acid might be part of the structure of that compound, and it was shown that biotin could be substituted for pimelic acid in the nutrition of *C. diphtheriae* (Allen) and was functional at lower concentrations.² It appears that when this strain is supplied with pimelic acid it can synthesise biotin.

It has already been mentioned that biotin is adsorbed on to egg white; the compound responsible for this effect is a protein, avidin, which can be used to render culture media biotin-free. Thus *C. diphtheriae* (Allen) when using biotin as a nutrient is inhibited by avidin, but not when using pimelic acid. In the course of chemical work on biotin it was transformed by hydrolysis to desthiobiotin. This compound can replace biotin in the nutrition of some organisms; thus it is functional for many strains of *S. cerevisiae*, *N. crassa* and *Leuconostoc mesenteroides*, but not for *Rhizobium trifolii* nor for *L. casei*. In those cases where it replaces biotin it does so at the same concentration, and at low concentrations the effect of the two compounds is additive. The evidence leaves little doubt that desthiobiotin is a stage in the normal biosynthesis of biotin. When the urea ring is ruptured the resulting diamino-carboxylic acid has only 10% of the activity of biotin; it is doubtful, therefore, whether this compound is an intermediate in the biosynthesis of biotin.³

Thus a nutritional demand for biotin may be due to inability to synthesise pimelic acid, or desthiobiotin, or both; the first is exemplified by *C. diphtheriae* (Allen), the second by *S. cerevisiae* and *Rh. trifolii*, the third by *L. casei*.

Desthiobiotin inhibits growth of those organisms which cannot substitute it for biotin.^{4, 5} This effect is abolished by increasing the concentration of biotin; 0.4 μg . biotin neutralises the effect of 2500 μg . of desthiobiotin whilst 2000 μg . of desthiobiotin is re-

¹ Mueller, 1937 (1), (2).

³ Melville *et al.*, 1943.

⁵ Lilly & Leonian, 1944.

² du Vigneaud *et al.*, 1942.

⁴ Dittmer *et al.*, 1944.

quired to antagonise 0.025 μg . biotin. The action of the former is therefore probably due to competition with those enzymes concerned in the utilisation of biotin. Such effects are frequent in the metabolism of exacting strains and this one is comparable with the inhibition of the metabolism of tryptophan by indoleacrylic acid.¹

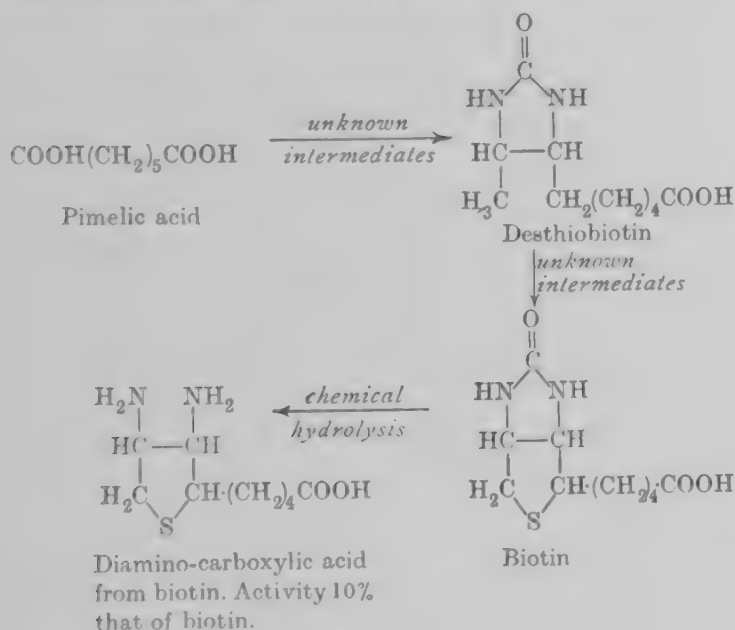


FIG. 21

Function of biotin

The function of biotin in the cell has been studied with a strain of yeast (*S. cerevisiae* Fleischmann 139) by the method of Lwoff, with important results which could hardly have been achieved except by the use of micro-organisms.²

Avitaminosis (biotin) in rats and chicks leads to severe skin lesions, emaciation and death; biotin deficiency in animals, including man, can be achieved only by the adsorption of the vitamin from the gut by egg white or avidin.³ In all animals so far tested skin symptoms are the first to appear, followed by acute malnutrition, none of which discloses the mode of action of the vitamin. Light on this has, however, been thrown by the use of a biotin-deficient yeast (*S. cerevisiae* Fleischmann 139) which requires biotin supplied in the medium.⁴ This organism attains normal growth with a biotin concentration of 3×10^{-2} $\mu\text{g./ml.}$; at a biotin level of 3×10^{-6} one-fifth maximal growth is obtained, giving biotin-deficient cells. An intermediate effect is obtained at a biotin level of 3×10^{-5} $\mu\text{g./ml.}$ Yeast grown at these levels

¹ Fildes, 1945.² Eakin *et al.*, 1941.³ Winzler *et al.*, 1944.⁴ Winzler *et al.*, 1944.

is referred to below as normal, deficient and intermediate respectively. When the metabolism of these three types of cell is examined it is found that deficient yeast has a respiration and fermentation rate of one-twentieth to one-tenth that of the normal yeast. These rates remain constant for many hours (see Fig. 22). I

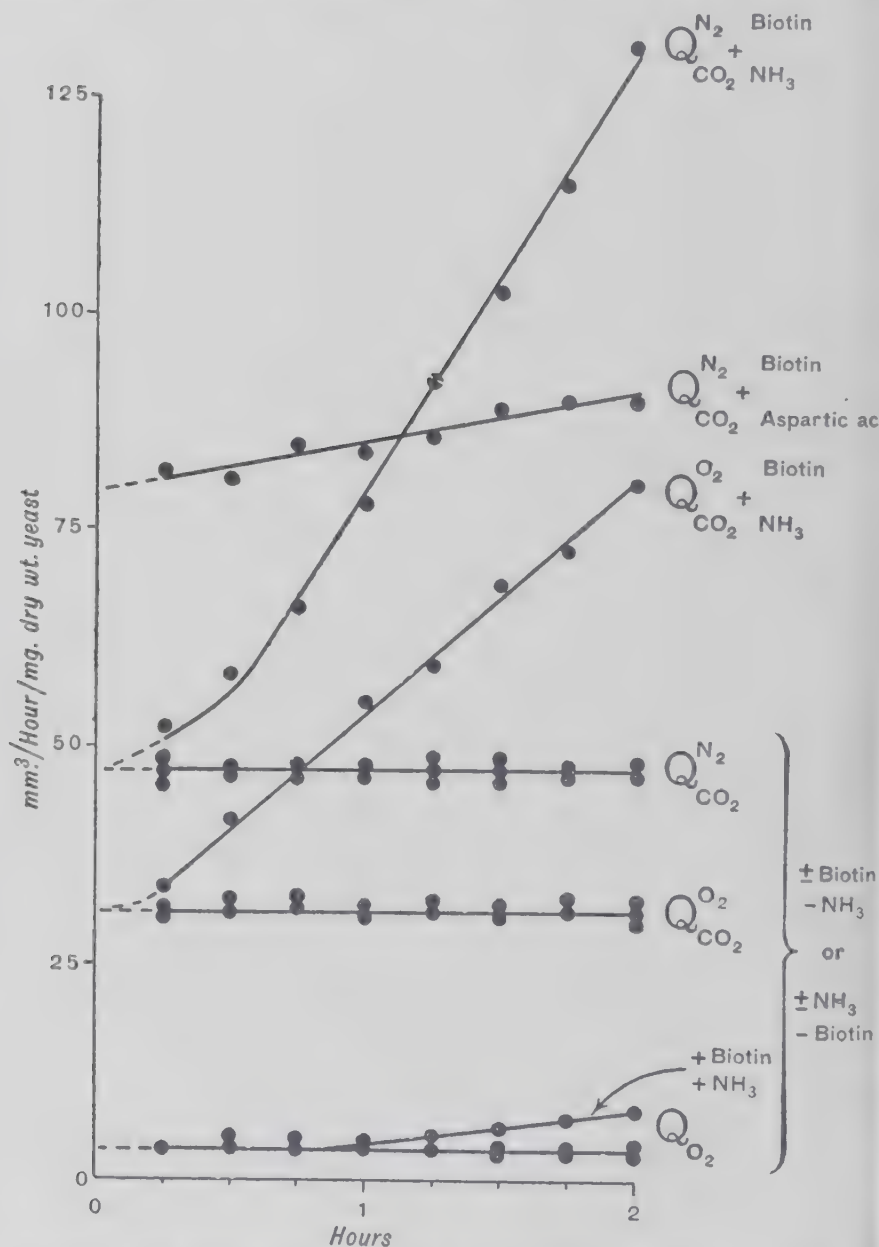


FIG. 22.—The effect of biotin, ammonia and aspartic acid on the respiration and fermentation of biotin-deficient yeast¹

¹ Winzler *et al.*, *Arch. Biochem.*, 5, 31 (1944).

biotin and ammonia are added an immediate increase in rate of both respiration and aerobic and anaerobic glycolysis occurs (see Fig. 22); biotin in the absence of ammonia and ammonia in the absence of biotin have no effect. If, however, ammonia is added from the start and biotin after 2 hours the curve is the same as if both are added together at the beginning, but when biotin is added from the start and ammonia after 2 hours the biotin effect is established more rapidly. This indicates that the presence of biotin in the cell preconditions it to the use of ammonia. Normal yeast shows no biotin effect when biotin and ammonia are added; and the same yeast when deficient in β -alanine or pantothenic acid—the only other vitamins to which it responds—shows no increase of fermentation rate on the addition of ammonia and either of these vitamins. Intermediate yeast gives a biotin effect between that of normal and deficient yeast. Sodium azide has no effect on anaerobic fermentation but inhibits carbohydrate synthesis; in concentrations of $M \times 10^{-4}$ and upwards it abolishes the biotin effect; at $M \times 10^{-5}$ the biotin effect is halved. Normal yeasts, able to synthesise biotin, give no biotin effect.

The biotin content of the cells growing in excess of and deficient in biotin was determined. The former contained $13.9 \mu\text{g.}$, the latter $0.017 \mu\text{g.}$ per g. yeast (dry weight), i.e. they differed by a factor of 800; the biotin content of normal and deficient yeasts was computed at 10^6 to 10^3 mols. per cell respectively.

Deficient yeast shaken with biotin and ammonia takes up its full quota of both in 2 hours; biotin is also taken up in the absence of ammonia but not in the absence of glucose. Ammonia uptake requires the presence of both biotin and glucose. The glucose probably supplies the necessary energy for the transfer of ammonia across the membrane whilst the assimilation of ammonia is also conditioned by biotin. These illuminating studies relating biotin activity to nitrogen metabolism probably mark an important milestone in the advance of knowledge of anabolic nitrogen metabolism.

p-Aminobenzoic acid



The discovery of *p*-aminobenzoic acid as a growth factor is of interest for two reasons: its importance in bacterial nutrition was recognised before it was known to be of importance to the animal, and its existence as a growth factor for micro-organisms was predicted before it was observed.

In an endeavour to find a "rational approach to chemotherapy," Fildes¹ put forward the view that chemotherapeutic agents may act either by combining with an essential metabolite or competing with it for a place on the surface of the enzyme which activates it. In the latter case it is necessary that the molecular structure of the poison should resemble that of the metabolite. Following this idea Woods² suggested the possibility that sulphanilamide might owe its chemotherapeutic action to competition with a molecule of similar structure and suggested *p*-aminobenzoic acid as a possibility. He was able to demonstrate that the inhibitory action of sulphanilamide on the growth of *Str. hæmolyticus* was antagonised by *p*-aminobenzoic acid in concentrations of 1/5000 to 1/25,000 that of the sulphanilamide; related substances were active only in higher concentrations (see Table 20), with the exception of novocaine, whose activity approached that of *p*-aminobenzoic acid.

TABLE 20³

ANTI-SULPHANILAMIDE ACTIVITY OF SUBSTANCES RELATED TO
p-AMINO BENZOIC ACID

Conc. of sulphanilamide = $3.03 \times 10^{-4}M$.

Substance	Active at <i>M</i> conc.
<i>p</i> -Aminobenzoic acid	$1.2-5.8 \times 10^{-8}$
<i>o</i> -Aminobenzoic acid	—
<i>m</i> -Aminobenzoic acid	0.9×10^{-3}
<i>p</i> -Nitrobenzoic acid	1.8×10^{-4}
Ethyl- <i>p</i> -aminobenzoate (benzocaine)	3.6×10^{-5}
Novocaine	5.8×10^{-8}
<i>p</i> -Hydroxybenzoic acid	—
<i>p</i> -Toluic acid	—
Benzoic acid	—
Benzamide	—
<i>p</i> -Aminobenzamide	1.4×10^{-6}
2-(<i>p</i> -aminobenzylamino) pyridine	0.9×10^{-3}
<i>p</i> -Aminophenol	+
Sulphanilic acid	—*

— indicates substance inactive at $10^{-3}M$.

† inhibits growth down to $3.6 \times 10^{-5}M$.

* inhibits growth at $10^{-3}M$.

Cell extracts having an effect similar to *p*-aminobenzoic acid were obtained from yeast,⁴ streptococci⁵ and *Br. abortus*,⁶ and *p*-aminobenzoic acid was finally isolated from yeast by Blanchard.

¹ Fildes, 1940.

² Woods, 1940.

³ Ibid.

⁴ Ibid.

⁵ Stamp, 1939.

⁶ Green, 1940.

⁷ Blanchard, 1941.

The reciprocal action of sulphanilamide and *p*-aminobenzoic acid suggested the importance of the latter in bacterial metabolism and caused Woods to predict that it would at some time appear as a bacterial growth factor. This was verified in the same year when it was found to be necessary to the growth of *Cl. acetobutylicum*;¹ since when it has been shown to be a necessary growth constituent for several bacterial species² as well as for an artificial mutant of *Neurospora*.³

The discovery of the antagonistic action of sulphanilic acid towards a growth factor with a configuration differing only in the replacement of a carboxyl by a sulphonic group suggested that active drugs might be synthesised by an extension of the same principle. This idea has been exploited by McIlwain,^{4, 5, 6} who has prepared analogues of nicotinamide, pantothenic acid and some amino-acids by replacing —COOH of the growth factor or amino-acid by $\text{—SO}_3\text{H}$. Fig. 23 shows examples of such analogues.

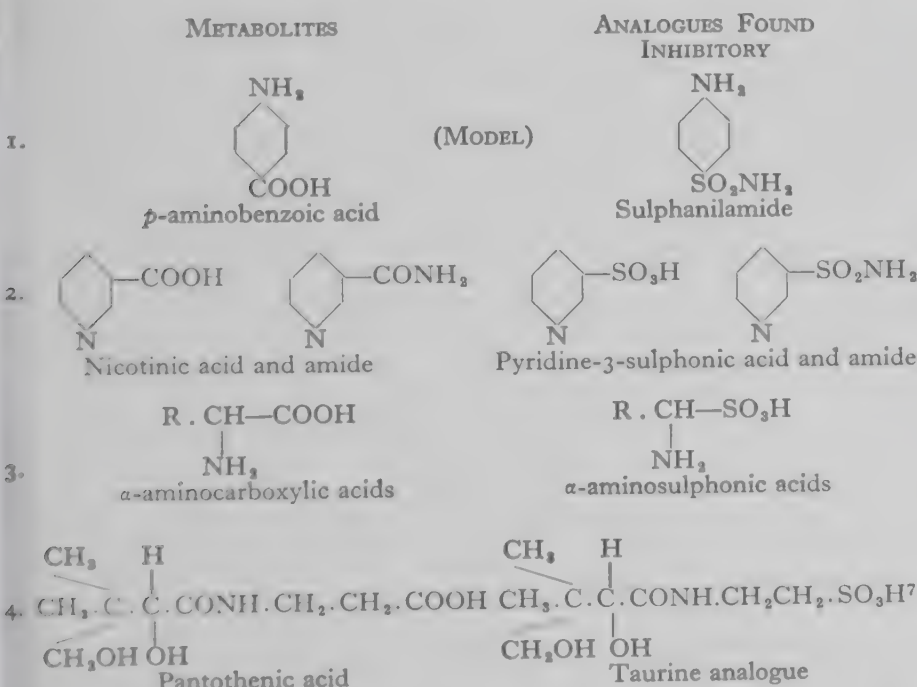


FIG. 23.—Applications of structural analogy with metabolites to the synthesis of inhibitors^{7, 8}

These exert an inhibitive action on the growth of those organisms exacting towards the growth factor or amino-acid structurally

¹ Bullock & Gillespie, 1940.

⁴ McIlwain, 1942 (1), (2).

⁷ Ibid., 1943 (1), (2).

² Knight, 1945.

⁵ Ibid., 1943 (1).

⁸ Snell, 1941.

³ Tatum & Beadle, 1942.

⁶ Ibid., 1943 (2).

related. Organisms able to synthesise the growth factor can become resistant to the drug by stepping up the synthesis of the factor it antagonises; exacting organisms are unable to do this. Further discussion of this subject belongs properly to chemotherapy, a field outside the scope of this book.

It may not be irrelevant to mention here that *l*-methionine also antagonises sulphanilamide as well as other sulphanilyl drugs.¹ The mechanism of this inhibition is discussed in the references given.^{2, 3}

In addition to its isolation from yeast, *p*-aminobenzoic acid has been shown to be present in a number of bacterial species which do not require it supplied in the medium,⁴ and it is now known to play a part in the nutrition of the chick,⁵ rat⁶ and man.⁷ It is a constituent part of the molecule of folic acid.

Choline

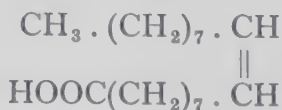


Choline has been shown to be a necessary growth factor for the pneumococcus (types I, II, V and VIII).⁸ In the case of type III a number of related compounds could be substituted without loss of activity and a number of others gave a very high response.⁹ According to Badger¹⁰ the most probable function of choline is in the synthesis of the phospholipids, e.g. lecithin, of which it forms part of the molecule. The role in transmethylation which it plays in animal tissues seems to be excluded by the activity of triethylcholine and diethylethanolamine and the inactivity of such methylating compounds as methionine, betaine and phosphoryl choline.

A mutant strain of *Neurospora*, however, shows different requirements; here lecithin could be substituted for choline and 50% of the choline available was used in three days. Here, however, methionine showed some activity—1/500 that of choline.

Substituted choline derivatives show much greater activity than is usually the case with growth factors,¹¹ several alkyl substitutions on the N atom giving full activity. Ethanolamine gave 80% of the activity of choline, which suggests that the function of choline is in the synthesis of phospholipids. The function of choline is fully discussed in the original paper¹² and by Knight.¹³

Oleic acid



Oleic acid is an essential nutrient for certain strains of *C. diph-*

¹ Harris & Kohn, 1941.

² Kohn & Harris, 1942.

³ Knight, 1945.

⁴ Landy *et al.*, 1943 (1).

⁵ Ansbacher, 1941.

⁶ Ibid.

⁷ Sieve, 1941.

⁸ Rane & Subbarow, 1938.

⁹ Badger, 1944.

¹⁰ Ibid.

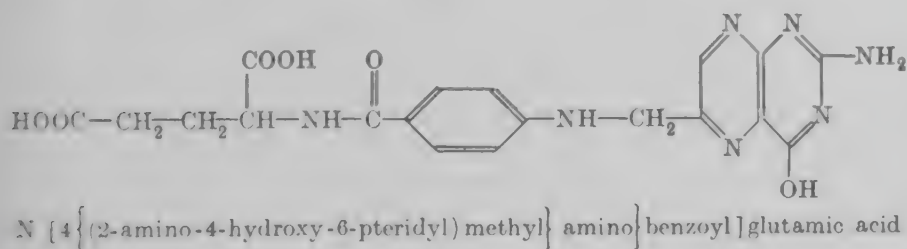
¹¹ Ibid.

¹² Ibid.

¹³ Knight, 1945.

theria, the optimum concentration being 0.05 mg./ml., also for *Erysipelothrix rhusiopathiae* and *Cl. tetani*. It has been shown that oleic acid (as well as linoleic and linolenic acids) exerts a temporary inhibition on *L. helveticus* and some other gram-positive organisms, lasting 24-48 hours at a concentration of 1.6 mg./ml.; the *trans*-isomer is without effect. This inhibition is reversed by lecithin, cholesterol, calciferol and α -tocopherol and its acetate. Gram-negative organisms tried did not show this inhibition.¹

Folic acid (the L. casei factor of liver)



A growth factor found originally in spinach and later in liver is one of the many factors necessary for the growth of *L. casei* and some other lactic organisms. With five strains of *Str. lactis* and three lactic bacilli, thymine and its nucleoside thymidine replace folic acid, but the effective ratio of thymine:folic acid is 5000, 3-4 μ g. thymine being required for the synthesis of 1 mg. cells. This suggests that folic acid is the prosthetic group of an enzyme concerned in the synthesis of thymine.²

Amino-acid requirements

Among synthetic disabilities acquired by many bacteria is that for the synthesis of amino-acids. As in the case of growth factors this may vary in number from one to ten or more, and is more commonly met with among gram-positive than among gram-negative organisms. It is also true that species with high vitamin requirements generally also are those most exacting towards amino-acids. Table 21 summarises the amino-acid requirements of six exacting organisms.

¹ Kodicek & Worden, 1944, 1946.

² Stokes, 1944.

TABLE 21

Amino-acid, etc.	<i>L. casei</i> ¹	<i>L. arabinosus</i> ²	<i>S. lactis</i> ³	<i>S. zymogenes</i> ⁴	<i>Leuconostoc mesenteroides</i> ⁵	<i>Cl. sporogenes</i> ⁶
Glycine . .	—	—	—	—	I	—
Alanine . .	(I)	—	(I)	(I)	—	—
Valine . .	I	I	I	I	I	(I)
Leucine . .	I	I	I	I	I	I
iso-Leucine . .	(I)	I	I	I	I	—
Serine . .	I	—	(I)	(I)	I	—
Threonine . .	(I)	(I)	—	(I)	I	—
Methionine . .	(I)	I	(I)	(I)	I	(I)
Tyrosine . .	I	I	—	(I)	I	I
Glutamic acid . .	I	I	—	I	I	—
Arginine . .	I	I	I	I	I	I
Lysine . .	(I)	(I)	(I)	(I)	I	—
Histidine . .	(I)	—	—	(I)	I	(I)
Tryptophan . .	I	I	—	I	I	I
Phenylalanine . .	I	I	(I)	—	I	I
Aspartic acid . .	I	(I)	—	—	I	—
Cystine . .	I	I	(I)	—	I	(I)
Proline . .	—	—	—	—	I	—
Glutamine . .	—	—	—	—	—	—
Asparagine . .	—	—	—	—	I	—

I indicates that the amino-acid is indispensable.

(I) indicates that the amino-acid improves growth.

Pyrimidines and purines

Compounds which have been most recently shown to be required by exacting organisms are pyrimidines and purines.

Uracil was shown by Richardson⁷ to be necessary for *Staph. aureus*, but only in anaerobic conditions when the organism became exacting also to pyruvic acid in a medium already containing glucose. Aerobically the organism was found to synthesise uracil. The demand for uracil is highly specific, a large number of related compounds having been proved useless. Uracil is also a necessary growth factor for an exacting dysentery organism, *Shigella paradysenteriae* (Flexner).⁸

Adenine, guanine, xanthine and hypoxanthine are important growth stimulants for *L. arabinosus*, *L. pentosus* and *Str. lactis*,⁹ and for *Str. haemolyticus* (group A) the factors appear to replace each other. Guanine and adenine are stimulatory for *L. casei*.¹⁰ In the case of *Str. haemolyticus* the purines are replaceable by their nucleotides and nucleosides.¹¹

¹ Hutchings & Peterson, 1943.

² Hegsted, 1944.

³ Niven, 1943.

⁴ Niven & Sherman, 1944.

⁵ Dunn *et al.*, 1945.

⁶ Fildes & Richardson, 1935.

⁷ Richardson, 1936.

⁸ Hutner, 1944.

⁹ Snell & Mitchell, 1942.

¹⁰ Feeny & Strong, 1942.

¹¹ Pappenheimer & Hottle, 1940.

Using *Str. haemolyticus* and adenylic acid as growth factor, a correlation was found between the adenylic acid and the CO_2 pressure. At high pressures of CO_2 (40 mm. Hg.) adenylic acid became unnecessary and its addition caused no increase of crop. In the absence of CO_2 no significant growth occurred in the absence of adenylic acid. The general relationships are obvious from Table 22.

TABLE 22¹

EFFECT OF ADENYLIC ACID AND CO_2 ON GROWTH OF *Str. haemolyticus*
C.203 S (277)

$\text{P.O}_2 = 120$ mm.

Total p. made up to 740 mm. with N_2 .

Growth in mg. bacterial N/500 ml.

CO_2 pressure mm. Hg.	Growth			
	No adenylic acid		Adenylic acid 10 $\mu\text{g.}/\text{ml.}$	
	20 hours	40 hours	20 hours	40 hours
0.0	—	0.3	—	2.2
0.4	0.0	0.7	1.9	6.8-8.2
1.4	0.1	0.7	3.1	8.4
2.4	0.15	3.8	3.0	9.1
4.3	0.15	9.0	10.9	10.1
8.0	1.4	9.1	11.3	10.4
20.0	1.0	9.2	9.6	10.8
40.0	10.0	12.2	12.2	12.4

The requirements of bacteria for these compounds are obviously important, though at present knowledge on this subject is very rudimentary.

Reconstruction of a complex medium

Having considered some of the individual requirements for bacterial growth, it is instructive to see how various units contribute towards supplying the optimum growth of any one organism. Such a study has been made by Mueller in the case of a difficult strain of the diphtheria bacillus.² The stock medium for this organism was Liebig's extract 0.5%; peptone 1%; and NaCl 0.5%. Growth at 35° was estimated by a micro-kjeldahl method at 55-60 hours. As a basis for fractionation, the stock medium was replaced by that given in Table 23. This gave a

¹ Pappenheimer & Hottle, *Proc. Soc. exp. Biol. Med.*, **44**, 645 (1940).

² Mueller, 1935.

TABLE 23

	mg./ml.
A. Salt mixture	
NaCl	5.0
K ₂ HPO ₄	0.15
CaCl ₂	0.0107
MgSO ₄	0.0107
FeCl ₃	0.0035
HCl conc. . . .	0.035
B. Liebig's extract	7.5
C. Tryptophan	0.1
D. Acid hydrolysate of caseinogen	10.0

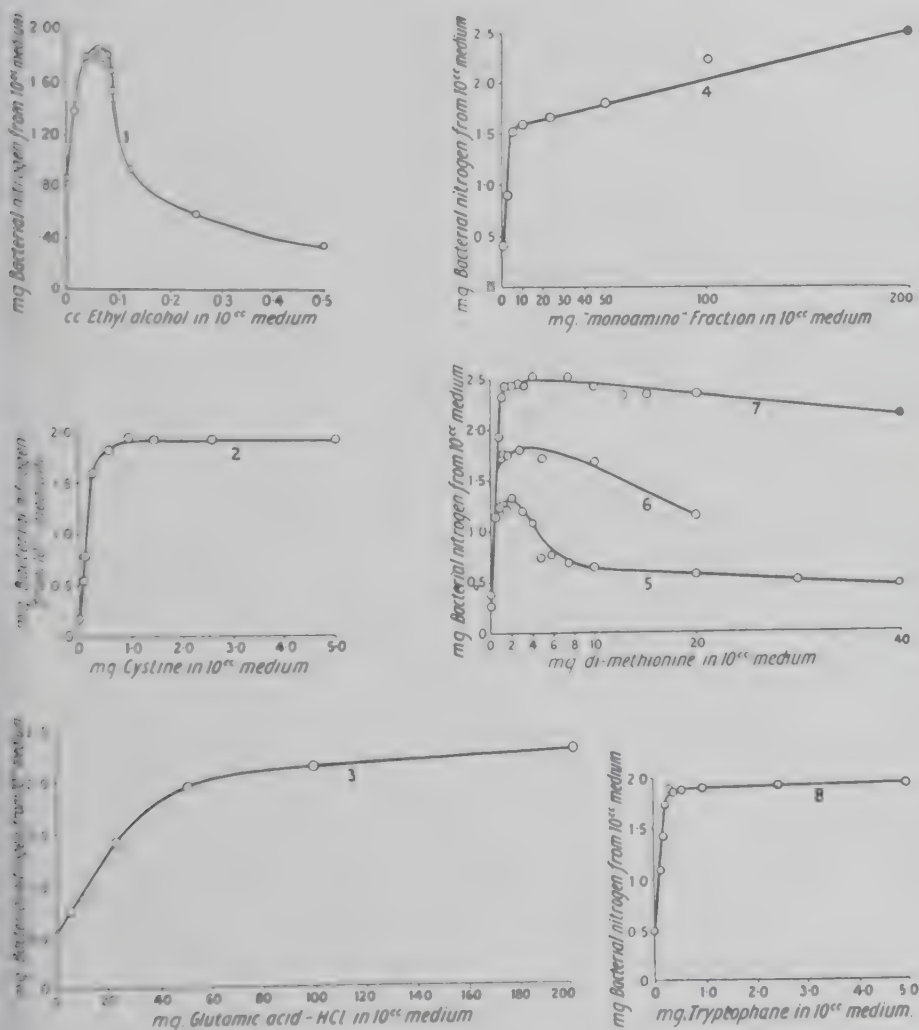
growth equivalent to about 0.2 mg. bacterial N/ml. A and B were left constant, C replaced the tryptophan destroyed by acid hydrolysis, and D was fractionated by Dakin's butyl alcohol extraction method, as shown in Table 24. Keeping A, B and C constant,

TABLE 24
ACID HYDROLYSIS OF CASEINOGEN, D

D-I (monamino fraction)	D-II (proline fraction)	D-III (unextracted residue)
(1) <i>l</i> -Methionine (2) <i>l</i> -Histidine (3) Glycine (4) <i>l</i> -Valine (5) <i>l</i> -Phenylalanine	(6) Ethyl alcohol	(7) <i>l</i> -Glutamic acid (8) <i>l</i> -Cystine

D was adequately replaced by D-I, D-II and D-III. Keeping D-I and D-III constant, D-II could not be replaced by proline and its activity was finally attributed to ethyl alcohol (6) present as a solvent in this fraction (see Fig. 24, curve 1). Keeping D-I and D-II (or 6) constant, the essential constituents of D-III were shown to be *l*-cystine (curve 2) and *l*-glutamic acid (curve 3). The former is adequate at 2 mg./ml. whilst the latter is required at 50-100 mg./ml.; this suggests that glutamic acid supplies not only a building stone, but a source of carbon and energy. Keeping 6, 7 and 8 constant, D-I showed a marked positive effect (curve 4). Of the nine amino-acids known to be present in D-I, glycine, alanine, valine, leucine, serine, phenylalanine, methionine, tyrosine and oxyproline were found to be dispensable, whilst suboptimal growth was obtained on methionine 0.14 mg./ml. alone. This compound, however, displayed a sharp optimum beyond which it had an inhibitory action (curve 5), a phenomenon not observed when it was replaced by the total monamino fraction D-I. The toxic effect of high concentrations of methionine partially disap-

peared when *l*-histidine (0.1 mg. ml.) was added (curve 6), and was entirely wiped out when all *dl*-phenylalanine (0.1 mg. ml.), glycine (0.5 mg. ml.) and *dl*-valine (1.0 mg. ml.) were also added (curve 7). Hence the acid hydrolysate of caseinogen is qualitatively and quantitatively replaced by items 1, 2, 3, 4, 5, 6, 7 and 8; tryptophan is seen to work at an optimal concentration of 0.2 mg./ml. (curve 8).

FIG. 24¹

The Liebig's meat extract was quantitatively replaced by nicotinic acid (1 μ g./ml.), β -alanine (0.1 μ g. ml.) and pimelic acid (0.01 μ g./ml.).

¹ Mueller, *J. Bact.*, 29, 522-7 (1935). Reproduced by permission of Williams, Wilkins Co., Baltimore, U.S.A.

Influence of media on fat formation

The growth of bacteria is influenced by the medium in a qualitative as well as in a quantitative direction. This has been demonstrated in the relative amount of lipid substances formed on varying sources of carbon. With respect, for example, to the Timothy grass bacillus, Stephenson and Whetham¹ showed that on an inorganic medium in which the nitrogen was supplied by ammonium salts (medium VII), the relative amounts of nitrogenous and lipid material were conditioned by the nature of the

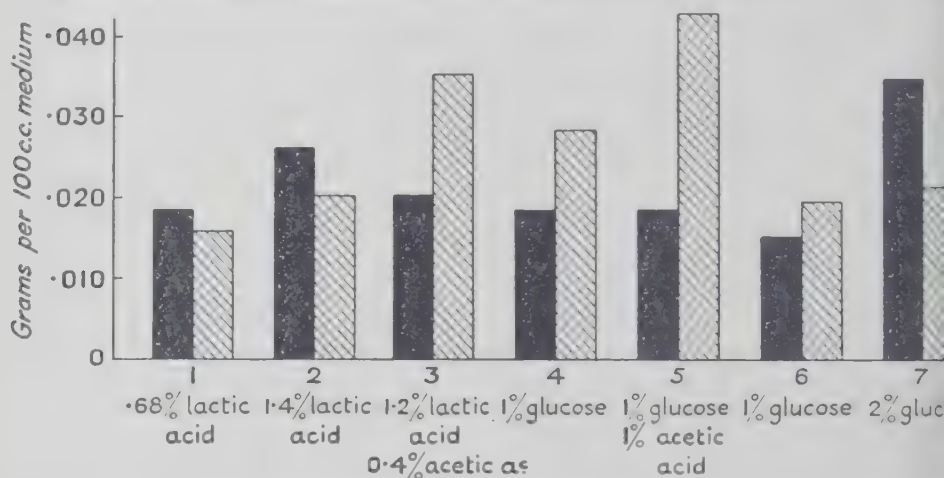


FIG. 25.—Diagram showing the influence of diet on the chemical composition of the Timothy grass bacillus

Black blocks represent grams of nitrogen synthesised per 100 c.c. of medium.

Shaded blocks represent grams of lipid synthesised per 100 c.c. of medium.

(Stephenson & Whetham, *Proc. Roy. Soc., B.*, 1922, 93, 278.)

carbonaceous food. The composition of the bacterial growth was taken when the lipid material was maximal, i.e. at the time just prior to the complete disappearance of the carbon compounds from the medium, and is given in Fig. 25. It is here seen that the proportion of protein (as measured by total nitrogen) to total lipid differs according to whether the carbon was supplied as lactate or as glucose; more striking is the addition of acetate to each. This addendum causes no increase in protein formation, but a high increase in lipid; increased concentration of lactate or of glucose, on the other hand, increases both protein and lipoids, with a preference for the former. The function of the lipid thus formed appears to be similar to that in the animal kingdom. On the final exhaustion of the carbon supply of the food, i.e. in starvation, the lipid content falls rapidly to about one-half, the fat fraction being

¹ Stephenson & Whetham, 1922.

burnt more quickly than the phosphatide, whilst the protein content remains steady.¹ The change in metabolism at the point when the carbon of the medium is exhausted and growth ceases is shown by the change in the respiratory quotient, which is higher than unity during the growth period and abruptly falls at the commencement of starvation to values indicating the combustion of fat.²

The influence of carbon compounds added to broth media has also been studied with interesting results; these are summarised in Table 25, taken from the work of Larson and Larson.³ As

TABLE 25⁴

Organism	Per cent of lipid extractives on		
	Plain broth	Broth + 0.5 per cent glucose	Broth + 3 per cent glycerol
<i>B. coli communis</i> . .	9.59	9.09	19.9
<i>Staph. albus</i> . . .	7.97	7.6	39.9
<i>B. megatherum</i> . . .	9.15	18.1	33.8
<i>B. mucosus</i>	13.9	8.82	15.42

the authors point out, the organisms which ferment glucose are unable to utilise it for fat formation, whilst the more aerobic and oxidising type (*B. megatherum*) achieves a twofold increase thereon. Similarly *B. mucosus*, which also ferments glycerol, actually suffers a decrease of fat formation as a result of the presence of glucose, probably owing to the deleterious effects of the acid formed.

Spore formation⁵

The conditions governing spore formation have not been closely studied, and generalised and misleading statements are frequently made. It is said, for example, that unfavourable conditions of temperature, acidity, accumulation of metabolic products and exhaustion of food supply all result in spore formation. Few of these statements bear rigid examination. Buchner,⁶ studying *anthrax* spores, stated that exhaustion of food supply was the prime cause of sporing, though none of his experiments were sufficiently rigid to exclude the possibility that change of reaction, accumulation of metabolic products and unsuitable salt balance might not be contributory or even primary causes. It is fairly clear that, in the case of this aerobe at any rate, spore formation does not occur in the absence of oxygen, and in the case of allied

¹ Stephenson & Whetham, 1922.

³ Larson & Larson, 1922.

⁵ Cook, 1932.

² Ibid., 1923.

⁴ Ibid., *J. Inf. Dis.*, 1922, 31, 407.

⁶ Buchner, 1890.

organisms it seems that liquid media containing glucose give rise to autolysis rather than to spores.¹

Fischhoeder's² observations on the behaviour of *anthrax* spores are very instructive. The spores were obtained by washing old cultures on nutrient agar with saline and killing of the vegetative forms by heating to 80° for 3 minutes. A second heating failed to decrease the viability of the culture, showing that the treatment completely differentiated between spores and non-resistant forms. Experiment showed that spore suspensions in saline kept at 37° do not germinate, though comparatively slowly. This was shown by removing duplicate samples *a* and *b* at intervals, and pouring *a* directly and *b*, after heating to 80° for 3 minutes, on to nutrient agar plates, and comparing the counts, the difference in the two counts representing the number of spores which had germinated in the interval. In physiological saline at 37° about half the spores germinated³ in 5 hours, in tap water the same proportion in 1 hour, whilst in broth an amazingly rapid change set in, about 98% of the original spores having passed to the non-resistant stage in 10 minutes.

It is improbable that the same factors influence the formation of spores in all cases, but there is evidence that in several instances nutrition plays a part. Henrici⁴ in the case of *B. cohærens* found that spore formation proceeded earlier on broth agar medium of low nutritive value and Williams⁵ noticed that spore formation with *Bac. subtilis* proceeded faster on 0.5% than on 5% peptone. Tarr⁶ showed definitely with five members of the *Bacillaceæ* that spore formation occurs most readily in low concentrations of nitrogenous nutrients. More detailed study of this effect is given in Table 26. There it is seen that dilution of casein broth 1/10 produces in all cases almost complete sporulation (media 1 and 3). The enrichment with Witte's peptone, ammonium phosphate and glycine has no marked effect, alanine, tryptophan and glutamic acid exert some inhibitory effect, whilst asparagine inhibits completely. It is noticeable that glycine inhibits growth but not spore formation.

Little is known about the germination of spores except in the case of the *Clostridia*. Here it has been shown in the case of *Cl. tetani*^{7, 8} that if the *Eh* of the medium is held constant by passing through it a stream of very dilute oxygen in nitrogen, a relation exists between the time required for spores to germinate and the O.R. potential at the electrode. The shortest germination time found was 4 hours at -0.05 volt or more negative; at more posi-

¹ Cook, 1931.

² Fischhoeder, 1909.

³ Henrici, 1928.

⁴ Ibid.

⁵ Williams, 1930.

⁶ Tarr, 1932.

⁷ Fildes, 1929.

⁸ Knight & Fildes 1930.

TABLE 26¹

% OF SPORES FORMED IN MEDIUM

Medium	1	2	3	4	5	6	7	8	9	10
<i>Strain</i>										
<i>B. mesentericus I</i>	1	96	98	85	93	98	7	61	83	84
<i>B. subtilis I</i>	3	89	97	77	80	*	0	75	94	83
<i>B. subtilis II</i>	0	91	96	56	98	82	0	44	81	70
<i>B. megatherum</i>	0	87	98	63	*	*	0	—	16	15

* Growth inhibited.

Media

Casein digest stock broth diluted with

1. 0.5% NaCl 1/3 containing 2.8 mg. amino N/ml.
2. " 1/15 " "
3. " 1/30 " "
4. " 1/30 + 1% Witte's peptone.
5. " 1/30 + 2% (NH₄)₂HPO₄.
6. " 1/30 + 2% glycine.
7. " 1/30 + 2% asparagine.
8. " 1/30 + 2% alanine.
9. " 1/30 + 2% tryptophan.
10. " 1/30 × 2% glutamic acid.

At more positive levels the lag increased so that at $Eh + 0.10$ volt it was 3-10 hours; at levels more positive than $Eh + 0.11$ volt (pH 7.0-7.65) germination never occurred, though spores remained viable.

¹ Tarr, H. L. A., *J. Hyg.*, 1932, **32**, 535.

CHAPTER VIII

NITROGEN FIXATION

THE faculty of using molecular nitrogen for the synthesis of cell material is, so far as we know, confined to micro-organisms, and is not shared by any of the more complex forms of life. It is true that periodically the power has been claimed for certain of the higher green plants, but so far it has always appeared subsequently that adherent symbiotic bacteria were the active agents. Indeed, even among bacteria only a very few species possess the faculty to any appreciable or useful extent.

The actual discovery that nitrogen fixation occurs through the agency of microbes was due to Jodin.¹ In a seldom-quoted paper published in 1862 he demonstrated that a solution containing phosphate, and either sugar, tartaric acid or glycerol, but no organic nitrogen, supported a vigorous growth of "mycodermis." If the culture fluid were enclosed in a sealed vessel, a decrease of nitrogen as well as of oxygen could be demonstrated, the nitrogen absorbed being 6-7% of the oxygen used. It is difficult to tell whether this important observation was influenced by Pasteur's writings on the microbial cause of fermentation; in any case, it stands as the first real biochemical evidence of the part played by microbes in nitrogen fixation. Subsequent to the early discoveries of Pasteur, an intensive study of soil problems took place. Thirteen years after Jodin's paper, Berthelot² demonstrated a rise in the organic nitrogen content of soils enclosed in pots and left uncultivated over a period of several months (increase due to soluble compounds of nitrogen carried down with the rainfall being duly allowed for). The fact that no increase occurred in soils previously sterilised by heat, or at the low temperature prevailing during the winter months, pointed to the agency of micro-organisms. A typical example of Berthelot's results is given in Table 1.

Later Berthelot³ obtained from the soil several organisms which, when grown *in vitro*, could be shown to multiply at the expense of atmospheric nitrogen, though whether he obtained any nitrogen fixers in pure culture is doubtful.

Meantime, Winogradsky⁴ had been attracted to the problem. He first showed that the inoculation of soil into a nitrogen-free

¹ Jodin, 1862. ² Berthelot, 1885. ³ *Ibid.*, 1893. ⁴ Winogradsky, 1893.

TABLE 1

NITROGEN PER KG. OF DRY SOIL (SANDY LOAM)

	30 April	6 July	10 October
Combined nitrogen, gm. .	0·1101	0·1279	0·1387
Nitrate " " .	0 0018	Traces	0·0009
Total " " .	0·1119	0·1279	0·1396
Gain of " " .	—	0·0160	0·0277

medium containing glucose resulted in a fixation of nitrogen coincident with an intense fermentation of the sugar. The following year he succeeded in isolating the microbe responsible, which proved to be an anaerobe (*Clostridium pastorianum*) whose apparent aerobic existence was due to the presence of contaminating aerobes. When grown anaerobically in a synthetic medium containing no combined nitrogen (No. 1) it was proved to multiply at the expense of atmospheric nitrogen, about 2 mg. of which appeared in organic form (as bacterial cell-stuff) per gm. of glucose fermented. The chief products of the fermentation were acetic and butyric acids, carbon dioxide and hydrogen.

Two other important observations were made by Winogradsky in the course of his work on *Clostridium pastorianum*. The first was that, under constant experimental conditions, the nitrogen fixed was closely proportional to the glucose broken down; this is shown in Table 2. The second was that the fixation of nitrogen

TABLE 2

	1	2
Glucose broken down, gm.	2·0	4·0
Nitrogen fixed, mg.	5·9	9·7
Mg. of nitrogen fixed per gm. of glucose broken down	2·9	2·4

was inhibited by ammonium salts (see Table 3, column 1). This inhibition could, however, be counteracted by increasing the concentration of glucose (see Table 3, columns 2, 3 and 4).

TABLE 3

	1	2	3	4
Glucose broken down, gm. . .	1·0	2·0	3·0	4·0
Nitrogen present, mg. (initial . . .	10·6	10·6	10·6	10·6
final . . .	10·6	11·4	14·3	14·7
gain . . .	0·0	0·8	3·7	4·1

If at the higher concentration of glucose the concentration of ammonium salts was again raised nitrogen fixation was again inhibited (see Table 4).¹

TABLE 4

	1	2	3	4	5	6
Glucose broken down, gm. .	3.0	3.0	3.0	3.0	3.0	3.0
Nitrogen present, mg. {	initial	4.2	6.4	8.5	17.0	21.2
	final .	9.1	9.2	11.9	12.1	17.3
	gain .	7.0	5.0	5.5	3.6	0.3

Winogradsky suggested that the fixation itself might involve the direct combination of nitrogen with nascent hydrogen produced in the breakdown of the glucose, ammonia being thereby formed. On such an assumption the inhibitive effect of ammonia would be explicable as the result of mass action; the removal of the inhibitory effect by the addition of more glucose would then be due to the removal of the ammonia to supply nitrogen for the increased growth of the cells so that the reaction $N_2 + 3H_2 \rightleftharpoons 2NH_3$ would then proceed in the \rightarrow direction.

Aerobic nitrogen fixation

The fixation of nitrogen is not, however, exclusively or even predominantly an anaerobic process, and is often carried out in the presence of oxygen gas. In 1901 Beijerinck isolated two similar organisms, one from both soil and canal water and the other a motile form found only in the latter, both of which when grown on his nitrogen-free medium (No. II) with a suitable source of carbon, were found to be capable of vigorous nitrogen fixation. The former (commoner) variety is known as *Azotobacter chroococcum*, the motile form as *Azotobacter agilis*.

The isolation of *Azotobacter chroococcum* is effected by following the method of Beijerinck.² The medium employed (No. II) with mannitol or propionate is used in preference to one with glucose, as the latter is more favourable to the development of *Clostridium pastorianum*. The medium is placed in thin layers in Erlenmeyer flasks, sterilised by steaming, and inoculated with 0.1 to 0.2 gm. of garden soil, and incubated at between 20° and 30°. After about three days the mixed culture which develops is subcultured into fresh medium and the process repeated two or three times. At this stage the culture is mainly *Azotobacter chroococcum*, and is able to fix nitrogen vigorously; other forms are, however, present, in particular a small bacillus, *Radiobacter*

¹ Winogradsky, 1894.

² Beijerinck, 1901.

which invariably occurs in the soil with *Azotobacter*, with which it lives symbiotically. *Azotobacter* can be obtained in pure culture by plating on to the same medium with 2% agar. Beijerinck originally asserted that when in pure culture *Azotobacter* no longer fixed nitrogen. Subsequent workers have disproved this, and plenty of results have been obtained of strong nitrogen fixation by *Azotobacter* in pure culture. It is generally agreed, however, that a somewhat more vigorous fixation occurs in mixed soil cultures. Beijerinck was also of opinion that traces of nitrogen compounds were necessary in order to start the growth of the organism, but this observation has not been corroborated.

The occurrence of nitrogen-fixing power

Though nitrogen fixation has been shown to be pre-eminently the property of a few species, yet evidence is not lacking to suggest that the line of demarcation between the fixers and other organisms is not so sharp as was at first supposed. It has already been mentioned that in certain media fixation is in abeyance and that the organism then grows at the expense of combined nitrogen; also that prolonged cultivation of *Clostridium pastorianum* in laboratory media results in loss of nitrogen-fixing power which by suitable cultivation can again be restored. That nitrogen fixation is not the exclusive property of the three groups of established nitrogen fixers has been made highly probable by a large amount of converging evidence; small amounts of nitrogen fixation by certain moulds,¹ and also by a few yeasts,² have been reported. The small degree of fixation in most of these cases makes it necessary to observe caution in accepting the results until corroborated by the use of isotopes.

Fixation by algæ

The blue-green alga *nostoc*^{3, 4} displays a high degree of nitrogen-fixing power. In synthetic medium (No. V) and no fixed nitrogen, with continuous illumination of 350 foot-candles and aeration with 1% CO₂ in air, growth and fixation run parallel, 9.8 mg. of nitrogen being fixed in 44 days. We have here an example of an organism "living on air." In the presence of glucose and absence of light fixation occurs at about twice this rate, amounting to 9 to 12 mg. per g. of sugar. See Table 5.

¹ Puriewitch, 1895; Ternetz, 1904; Fröhlich, 1907; Stahl, 1911.

² Lipman, 1911.

³ Allison & Hoover, 1935.

⁴ Allison, Hoover & Morris, 1937.

TABLE 5¹

	mg. N fixed in 44 days	
	Un aerated	Aerated with 1% CO ₂ in air
Control; basal medium only	1.5	5.3
Basal medium + CaCO ₃ 20 mg./100 c.c. . .	2.0	7.1
" " " 100 mg./100 c.c.	2.1	9.8
" " " 100 mg./100 c.c. + 1% sucrose	2.2	11.8

Distribution of nitrogen fixers in the soil

Azotobacter and *Clostridium* are widely distributed in soils, the former to a depth of about 50 cm., the latter reaching lower. They are also found in salt and fresh water, frequently in symbiosis with algæ. They are stated to be absent from virgin soils and from soils at high altitudes except where algæ are present. The presence of calcium carbonate favours their multiplication, probably by neutralising the acid products of carbohydrate breakdown and phosphates are essential. *Azotobacter* is not prevalent in peaty soils, as the acid reaction is unfavourable. Soils which fail to give a growth of *Azotobacter* when inoculated into selective culture media may do so when $5 \times 10^{-5}\%$ of sodium molybdate is added.² The increase of nitrogen fixers in the soil can be induced by dressings of lime, chalk, phosphate, and by carbohydrate refuse (molasses, etc.), according to the natural deficiency of the soil in question.

Symbiosis between *Azotobacter* and higher plants frequently occurs. *Chlorella*, for example, can develop in tap water when associated with *Azotobacter*, the former assimilating carbon dioxide and the latter pure nitrogen.³ Both *Azotobacter* and *Clostridium pastorianum* occur on the mucilaginous surface of seaweeds,⁴ and it is extremely probable, though not actually proved, that they exercise there a symbiotic function. The growth of green algæ in media free from combined nitrogen is probably due to a similar symbiosis, and not to nitrogen-fixing powers of the algæ, as suggested by Moore and Webster.⁵

The symbiotic nitrogen fixers

The observation that leguminous plants enrich the soil and prepare it for the growth of cereals has been handed down from early times; the fact, however, that these plants actually absorb

¹ Allison & Hoover, 1935.

² van Niel, 1935, (2). ³ Lipman & Teakle, 1925.

⁴ Issatchenko, 1926.

⁵ Moore & Webster, 1920.

atmospheric nitrogen was first proved in 1838 by the experiments of Boussingault.¹ This observer cultivated both clover and wheat in sterile sand, and showed that whilst both plants gained carbon, hydrogen and oxygen, only the former gained nitrogen (Table 6).

TABLE 6
THREE MONTHS' CULTURE

Plant	Gain in weight, g.				
	Total	Carbon	Hydrogen	Oxygen	Nitrogen
Clover .	2.574	1.276	0.176	1.026	0.042
Wheat .	1.378	0.689	0.078	0.608	0.003

Later experiments on very completely ignited sand failed to show a gain in nitrogen by either plant. Subsequent workers laid some stress on the presence of root nodules, and showed that these were not formed by seedlings grown in sterile soil or sand.² The nodules were also known to consist largely of bacteria, but the early tendency was to regard them as pathogenic rather than symbiotic in character.

In 1888 Hellriegel and Wilfarth,³ in a paper embodying the results of four years' experimentation, and justly regarded as classical, finally settled the relationship between nodule formation and nitrogen fixation. They demonstrated by means of pot culture in sand that whereas the *Graminaceæ* are dependent on combined nitrogen (nitrates) for growth, peas and other *Leguminosæ* can develop in its absence, and that in such cases the total nitrogen of the grown plant exceeds that initially present in the seed and the soil. In soil sterilised by heat and watered with sterile water, on the other hand, the *Leguminosæ* behave like the *Graminaceæ*, i.e. they depend on the presence of combined nitrogen. When instead of sterile water extract of agricultural soil was used for watering, the difference between the two types of plant once more manifested itself, i.e. the *Leguminosæ* developed but not the *Graminaceæ*.

Growth of the *Leguminosæ* and nodule formation were associated as follows :

Growth in sterile sand without nitrogen was stunted and curtailed, and no nodules appeared. Growth in sterile sand with nitrate was normal, but again no nodules were formed. In non-sterile soil nodules were produced both in the presence and absence of soil nitrate, their formation being accompanied by a gain in nitrogen, i.e. the nitrogen of the grown plant > the residual

¹ Boussingault, 1933.

² Frank, 1879.

³ Hellriegel & Wilfarth, 1888.

nitrogen of the soil exceeded the initial nitrogen of the seed + that of the soil. Thus the ability to use atmospheric nitrogen, whether in the presence or absence of soil nitrogen, never occurs without the presence of nodules: nodules never occur except in unsterilised soil, or by the addition of unsterilised soil extracts. Hellriegel and Wilfarth hence deduced that nitrogen fixation is due to a "soil ferment" whose *locus operandi* is the root nodules.

Later Schloesing and Laurent¹ demonstrated, by the volumetric analysis of the enclosed air in which peas with nodules were growing, that an actual absorption of nitrogen occurred whilst simultaneously the system soil and plant gained in nitrogen, an approximate agreement between the two being demonstrated (Table 7).

TABLE 7

	Exp. 1, mg.	Exp. 2, mg.
Loss of N from the air	36.5	32.5
Gain of N by plant and soil . .	40.6	34.1

Beijerinck² was the first to succeed in cultivating the nodule organism (*B. radicola*, now known as *Rhizobium*) apart from the plant. For this purpose he found ordinary laboratory media (broth, etc.) unsuitable, but attained success by the use of a decoction of leaves from leguminous plants with 0.25% asparagine and 0.25% cane sugar. He also demonstrated that the organisms occur free in the soil as well as in the nodules, thus accounting for the success of Hellriegel and Wilfarth in infecting the roots of plants growing on sterile sand by watering with aqueous extract of soil.

Nodule formation in other plants

Although symbiotic nitrogen fixation occurs most successfully in the *Leguminosæ*, yet nodule formation is met with in other orders sometimes but not always associated with nitrogen fixation. Many species of *Alnus* (alder), for example, have large root nodules. Hiltner³ first demonstrated the probability that these were concerned with nitrogen fixation by growing seedlings of *Alnus glutinosa* in sterile soil without nitrogen, some pots being inoculated with material from *Alnus* nodules. At the end of one year the plants in the inoculated pots were large and healthy, those in the uninoculated pots being small and sickly, with symptoms of nitrogen starvation. More recently similar but

¹ Schloesing & Laurent, 1890.

² Beijerinck, 1888.

³ Hiltner, 1896.

more detailed experiments on the same species have been carried out at Helsingfors.¹ Seeds of *Alnus glutinosa* were sown in aerated water cultures or in sand (a) without fixed nitrogen but inoculated with nodule material from the same species; (b) with ammonium nitrate and without inoculation; (c) without fixed nitrogen or inoculation. (c) gave no growth and (a) gave markedly better growth than (b) (this is unlike the case of the *Leguminosæ* where growth on fixed nitrogen is generally as good as and sometimes better than that dependent on nitrogen fixation). Nodules were formed only on plants in inoculated cultures and the superiority of the (a) over the (b) series held at all values of pH from 4.0 to 7.0. This series of experiments also shows a marked pH optimum of 6.0 for the (a) series; no effect of pH within the range tried was noticeable with the (b) series. A number of organisms were isolated from the *Alnus* nodules but none were effective in causing nitrogen fixation, though some caused nodule formation.

Another case of root nodules associated with nitrogen fixation is that of *Coriaria japonica* A. Gr.² studied by Kataoka. In several members of the *Elæagnaceæ* and in *Ceanothus* and *Myrica* nodules have been described without nitrogen-fixing function being definitely established.

Symbiotic nitrogen fixation in leaves

Bacteria in knots or nodules on leaves have been found in the *Rubiaceæ*³ and in *Dioscorea*.⁴ The organism from the former was cultivated in synthetic medium by van Faber⁵ and its nitrogen-fixing powers demonstrated; 5-6 mg. of nitrogen was fixed in 20 days on 200 ml. of a synthetic medium containing 2% gum-arabic as source of carbon. Faber also succeeded in obtaining plants free from the organism by careful sterilisation of the seeds; the growth of these was stunted as compared with those infected with the organism. In nitrogen-free sand the infected seedlings developed, whereas the sterile seedlings died off at an early stage. Successful inoculations of sterile seedlings with the organism were carried out, nodule formation and increased growth resulting.

The adaptation of manometric technique to the study of nitrogen fixation

The difficulty of measuring nitrogen fixation except by the tedious method of estimating nitrogenous cell material retarded progress in this field for a long time. The adaptation of the manometric technique to this problem (due to Burk and his school)

¹ Vartanen & Sastamoinen, 1936.

² Zarabekian, 1962.

⁴ Ott, 1923.

² Kataoka, 1930.

⁵ van Faber, 1912 and 1914.

has been mainly responsible for the progress achieved since 1927. The difficulty of measuring directly the uptake of nitrogen in fixation by *Azotobacter* is due to the fact that the process does not occur anaerobically. Hence in manometric measurement two changes of pressure are being measured simultaneously, one due to respiration and the other due to nitrogen fixation, the latter being relatively very small. The respiration rate of *Azotobacter*^{1,2} in the presence of carbohydrate or similar food material and at oxygen pressure of 20% of 1 atm. is extremely high ($Q_{O_2} = 2000$ or more). In such circumstances the pressure change due to nitrogen fixed is about 1% of the total gas change. However, by working at a reduced oxygen pressure (initially 2.1% oxygen in nitrogen) over a period of 14 hours it was possible to show an absorption of nitrogen as well as of oxygen.³ In Exp. A the manometer contained a dilute culture of *Azotobacter* in inorganic medium with 1% glucose and 0.1 mg. of ammonia-N per c.c. In Exp. B no ammonium salt was present (see Table 8). This experiment

TABLE 8

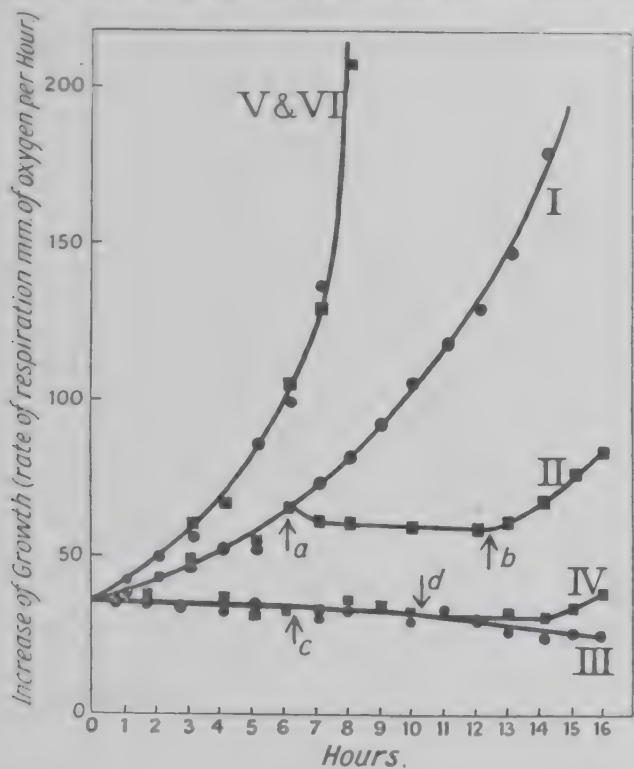
	A	B
Manometer reading	— 232	— 239
	— 232	— 249
	— 230	— 236
	— 230	— 239
Average	— 231	— 240.8

$$\frac{N_2 \text{ decrease}}{O_2 \text{ decrease}} = 4.2\%$$

shows that with carefully arranged conditions it is just possible to demonstrate nitrogen fixation directly by manometric means, but for practical purposes the method has little advantage over the Kjeldahl estimation of bacterial nitrogen. The successful adaptation of manometric technique to the measurement of nitrogen fixation is due to the circumstance that in young cultures of *Azotobacter* growing in inorganic medium with glucose and no fixed nitrogen the growth curve as measured either by cell count, dry weight or nephelometrically is reasonably superimposable on the respiration rate curve. Hence rate of respiration can be used as a measure of cell growth, which (in the absence of fixed nitrogen) means nitrogen fixation. This is illustrated in Fig. 1. The graphs in Fig. 1 represent the respiration of a 48-hour culture of *A. vinelandii* in inorganic medium (No. IV) and 1% glucose. Each vessel contained at the beginning 3×10^6 cells. Vessel III was filled with 21% oxygen in hydrogen. Here the respiration rate

¹ Meyerhof & Burk, 1928.² Lineweaver, 1932.³ Burk, 1930.

remained approximately constant for 6 hours, falling very slightly with age. At this time (*c*) the gas was changed to 1% nitrogen in 21% oxygen in hydrogen; no measurable growth was obtained after 10 hours more. The nitrogen was then raised to 6% (*d*);

FIG. 1¹

the respiration rate increased after a further 3 hours (IV). Curve I shows the rising respiration rate of a similar culture in air. Curve II shows the effect of changing the gas mixture (at *a*) to 21% oxygen in hydrogen, when growth stops (*a-b*). At *b* the gas mixture was changed to 20% nitrogen and 21% oxygen in hydrogen; growth immediately starts. Curves V and VI show the growth rate when fixed nitrogen is present as ammonia (0.10 mg. of nitrogen per ml.). Gas mixture in V is 21% oxygen in nitrogen; in VI, 21% oxygen in hydrogen. Here the growth rates are identical, showing that this concentration of ammonia suffices to inhibit fixation.

This rapid method of estimating nitrogen fixation has made it possible to study the process in detail and some results obtained will now be considered.

The measurements embodied in Fig. 1 were made in an atmosphere in which hydrogen was the diluent gas on the assumption that it was inert with respect to fixation. It is now known that it

¹ Burk, 1930, *J. phys. Chem.*, 34, 1180.

is a specific inhibitor for the fixation process, and hence the actual form of the curves obtained is influenced by this fact; nevertheless the experiment is valid in that it demonstrates that the respiration rate can be used as a measure of nitrogen fixation. Unfortunately, however, the use of hydrogen as a diluent has invalidated Burk's figures on the effect of oxygen and nitrogen pressures on rate of fixation, and figures obtained by later workers must be substituted.

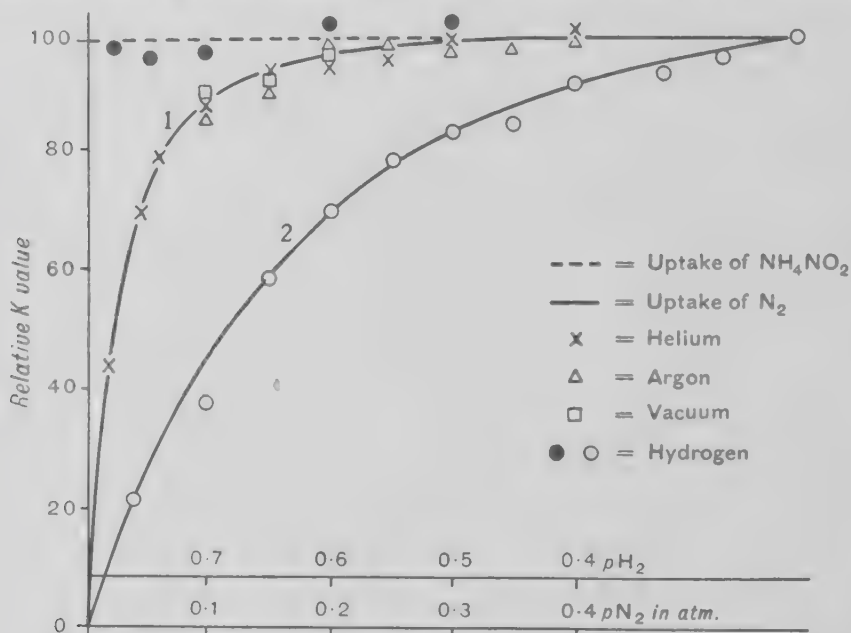


FIG. 2.—The pN_2 function of *Azotobacter* based on total N showing rate of fixation in different gas mixtures. The points shown are the means of 3–7 estimates¹

If the rate of fixation is measured at different pressures of N₂ diluted with helium or argon or carried out *in vacuo*, Curve I, Fig. 2, is obtained. Here it is seen that the rate of fixation remains constant till the pressure of N₂ is reduced to 0.15 atm.; it then rapidly declines, attaining 50% maximum rate at 0.01 atm. The effect of pH on fixation and on growth in fixed nitrogen is shown in Fig. 3. Growth in free nitrogen decreases from a maximum at pH 7.8 to zero at pH 5.97; irreversible inhibition takes place below pH 5.0.

Calcium and strontium are essential for fixation and also for growth on fixed nitrogen. Maximum effects are obtained by 2×10^{-4} and half maximum by 3×10^{-5} M Ca; strontium requirements are slightly higher. Where these elements are deficient the effect of adding them is observable in a few minutes

¹ Wyss *et al.*, *Biochem. J.*, **35**, 847 (1941).

to one hour, but the pH limit is unaffected by them. In accordance with the indispensability of calcium and strontium, growth is completely inhibited by oxalate $0.0015\text{ }M$ and 50% by $0.0005\text{ }M$.

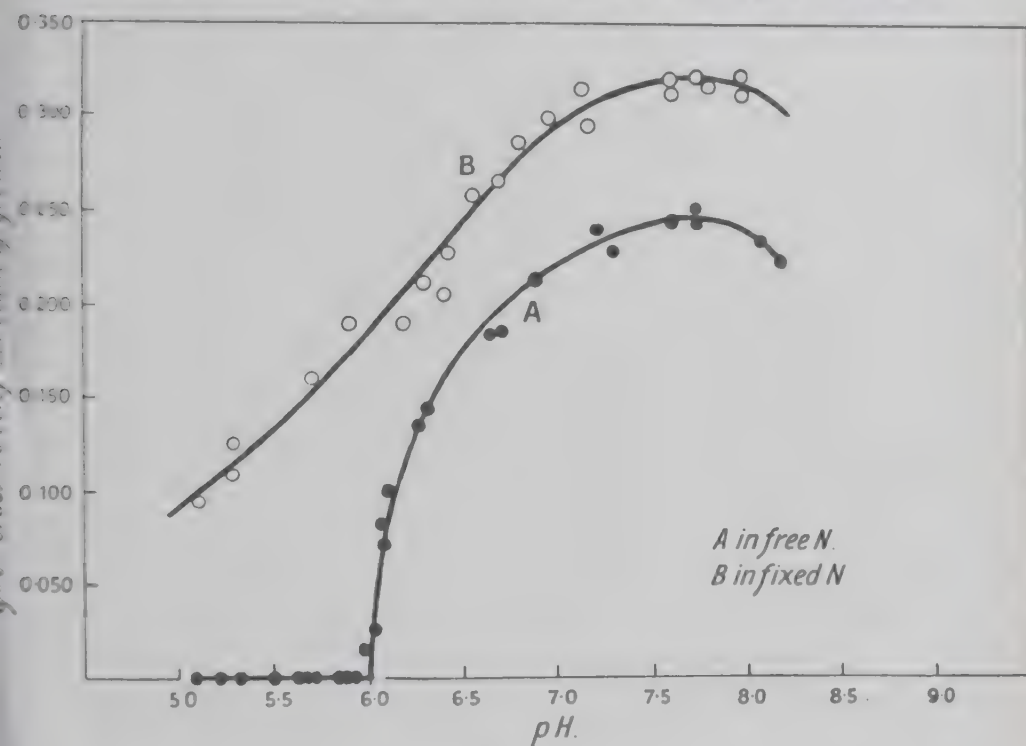


FIG. 3

Fluoride $0.0273\text{ }M$ is equivalent to oxalate $0.0015\text{ }M$ in ability to inhibit growth; this is roughly in accordance with the solubilities of calcium oxalate and calcium fluoride.^{1, 2, 3}

The effect of iron and magnesium

The beneficial effect of soil or humus on *Azotobacter* has been noted by several workers. Rosing,⁴ for example, showed that nitrogen fixation was markedly improved by acid extract of soil and that the extracted soil was inactive. The activity of the extract was shown to be due to iron and could be supplied by ferric oxide or phosphate equivalent to 15 mg. of Fe_2O_3 per 100 ml. of medium. Humus was shown by Burk and co-workers⁵ to increase the growth of *Azotobacter* on free and fixed nitrogen; it was again shown that this action was due to iron and could be replaced by iron salts. The alleged specific action of iron on fixation apart from growth appears to be illusory⁶ and possibly due

¹ Burk & Lineweaver, 1931.

² Burk, 1934.

³ Burk & Horner, 1940.

⁴ Rosing, 1912.

⁵ Burk, Lineweaver & Horner, 1932.

⁶ Burk, 1934.

in some cases to the presence of molybdenum. Humates which contain traces of a number of metals probably exert their effect through the non-specific influence of iron and the specific action of molybdenum.

Effect of molybdenum and vanadium

The beneficial effect of molybdenum was first observed by Bortels,¹ who found that it increased growth on free nitrogen two- to threefold. The most recent studies on this subject show that a detectable effect on growth of *Azotobacter* on free nitrogen is obtained at a concentration of molybdenum of 0.0001 p.p.m. and an optimal effect at 1 p.p.m.; 0.03 p.p.m. gives half maximal growth and 0.1 p.p.m. gives 80–90% maximal growth. Molybdenum is replaceable by vanadium at practically the same concentrations.² Certain strains of *Azotobacter* appear to be able to make more effective use of molybdenum than the majority and show only a twofold increase when molybdenum is added to the deficient medium, whereas most strains respond by a thirty- to forty-fold increase. This appears to be due to a more effective response to traces of molybdenum present in the control medium.³ Molybdenum has also been shown to have a favourable effect on fixation by the blue-green algæ.⁴

The extremely low concentrations of molybdenum specifically catalysing the fixation process led to some interesting speculations by Burk.⁵ It is seen that 10^{-9} *M* molybdenum (100 p.p.t.) is sufficient to give practically maximum fixation in a culture containing $2-6 \times 10^{10}$ cells per litre. This corresponds to $10^{-9} \times 6 \times 10^{23}$ molecules/ 6×10^{10} cells, that is, 10,000 molecules molybdenum per cell; 2000 molecules molybdenum per cell gives half maximum velocity. One cell of *Azotobacter* 2 μ in diameter contains about 10^{11} molecules, most of which are water, so 10,000 molecules per cell gives a ratio of 1 molecule molybdenum per 10^7 cell molecules.

Energy relations in fixation processes

In considering the possible mechanisms by which nitrogen may be fixed, the question of the free energy available in different reactions becomes very important. It is frequently assumed in the literature that nitrogen fixation must necessarily be an endothermic process, plausibility being lent to this assumption by the fact that in all the cases as yet described it is attended by the exothermic breakdown of organic substances. Burk,⁶ however, has investigated the subject from the standpoint of thermo-

¹ Bortels, 1930.

⁴ Bortels, 1940.

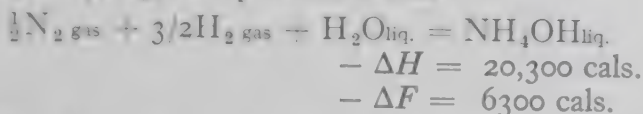
² Horner *et al.*, 1942.

⁵ Burk, 1934.

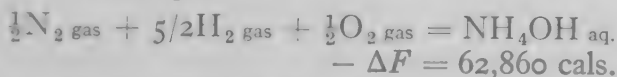
³ Ibid.

⁶ Ibid., 1927.

dynamics and has pointed out that several possible processes are both exothermic and yield free energy; for example, the production of ammonia from nitrogen and hydrogen (in standard conditions, see p. 13) is represented thus:

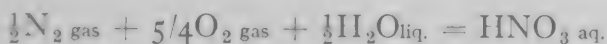


Supposing the reaction to occur by means of free hydrogen and free oxygen, as might be the case aerobically in the presence of formic acid ($\text{HCOOH}_{\text{aq.}} = \text{CO}_2 \text{ gas} + \text{H}_2 \text{ gas} - \Delta F = 6340 \text{ cal.}$), more free energy would be available owing to the replacing of water with oxygen gas,



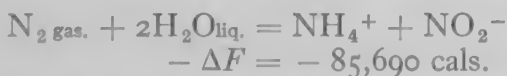
Burk has also suggested that the hydrogen normally present in the atmosphere, amounting on an average to 0.01% by volume, may conceivably play a part in nitrogen fixation by some organisms.

Although recent work renders it highly probable that fixation occurs by a reduction process, the possibility that oxygen may play a part cannot as yet be ruled out. Burk has calculated that though the reaction



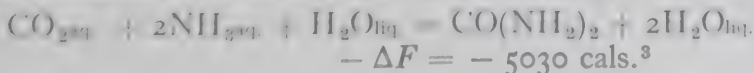
has a negative value for $-\Delta F$ under standard conditions (see p. 13), yet if the concentration of the HNO_3 is reduced to 0.1 *M*, then $-\Delta F$ is zero, this being the equilibrium concentration; further reduction in the concentration of HNO_3 gives a positive value for $-\Delta F$.¹

Another possible fixation process is that of the "hydrolysis" of nitrogen, i.e. the reversal of the denitrification reaction; this, however, in standard conditions, occurs with the absorption of free energy:



According to Lewis and Randall,² a pressure of nitrogen of 10^{51} atmospheres would be required for the formation of ammonium nitrite at the equilibrium concentration ($-\Delta F = 0$); hence if fixation occurs by this process energy must be supplied by some other reaction.

The formation of urea offers another possibility:



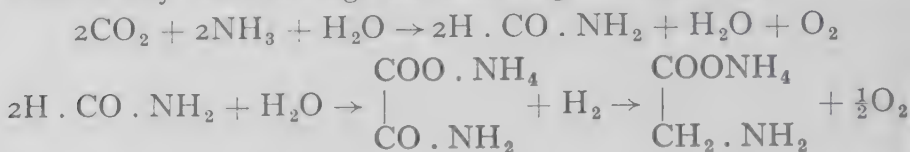
¹ Lewis & Randall, 1923, p. 538.

² *Ibid.*, p. 597.

³ Burk, 1927.

By decreasing the concentration of urea, however, the reaction may proceed without the addition of free energy; whilst if urea is formed from its elements — ΔF is positive (47,280 cal.).¹

It has been suggested by Löb,² on the analogy of syntheses obtained under the influence of the silent electric discharge, that fixation may occur through the following reactions:



No biochemical evidence, however, exists for this transformation, which is endothermic in character.

It is fairly clear that, in the presence of hydrogen arising from the fermentation of sugars or formate, nitrogen fixation can proceed without any associated energy-giving process, and that the same is true if the reaction is an oxidative one occurring in the presence of free oxygen. The older literature is, however, permeated with the idea that it is a process requiring the addition of energy, and many of the facts accumulated concerning the most favourable conditions for nitrogen fixation have been interpreted in that light.

The mechanism of nitrogen fixation

In spite of much study the actual mechanism of fixation, in particular the primary reaction by which N_2 enters into combination, is still obscure, but much evidence has recently accumulated.

It has been shown that molecular hydrogen inhibits fixation both in symbiotic fixation³ and with *Azotobacter*.⁴ This was shown in the former case by growing inoculated red clover plants in free nitrogen diluted with varying concentrations of H_2 . Controls using fixed nitrogen were also made. Batches of 20 plants were grown (a) on free, (b) on fixed nitrogen, at different partial pressures of H_2 , and harvested at intervals. The nitrogen assimilated by the plant was estimated. The results of this beautiful experiment are shown in Fig. 4. It is seen that the growth (which is logarithmic) in all cases is severely restricted by H_2 when dependent on N fixation, but that it is unaffected when occurring on NH_4 salts.

A similar effect was obtained with *Azotobacter*.⁵ In an atmosphere of N_2 fixation proceeds at the maximum rate till the pressure is reduced to about 0.15 atm., below which it falls rapidly. At 0.01 atm. the K value is about 50%. In the presence of H_2 a

¹ Lewis & Randall, 1923, p. 586.

⁴ Wyss *et al.*, 1941.

² Löb, 1913.

⁵ *Ibid.*

³ Wilson *et al.*, 1938.

significant reduction in the rate of fixation occurs at pN_2 0.45 atm. with a pH_2 0.35 atm. As the proportion of H_2 is increased the rate drops rapidly, so that at pN_2 0.05 atm. and pH_2 0.75 atm. fixation is barely detectable (see Fig. 2, p. 230).

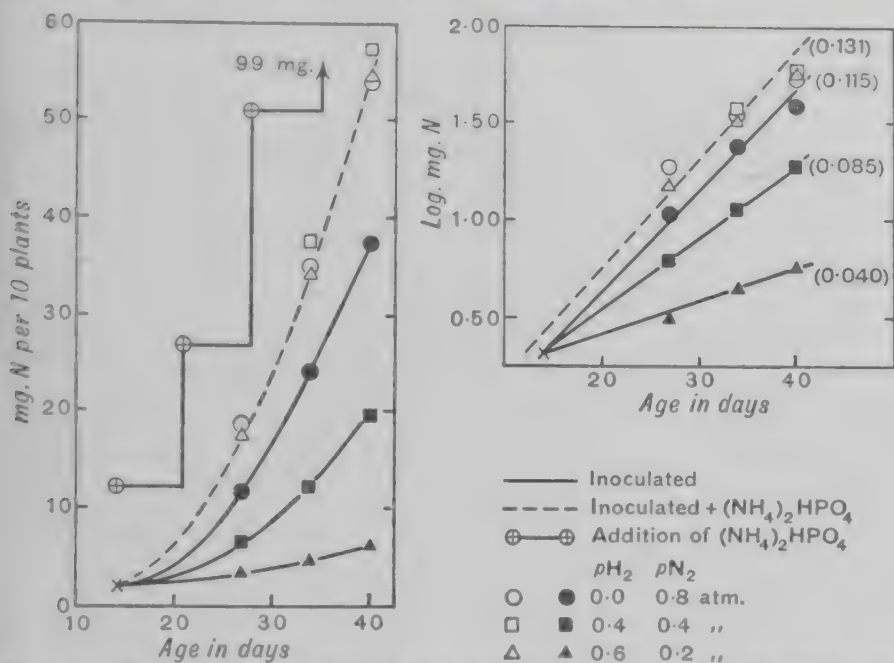


FIG. 4.—Effect of pH_2 in atmosphere on assimilation of free and combined N((NH₄)₂HPO₄) by red clover. (Exp. 2, planted 6 July 1937; all in air until harvest I, 20 July, then changed to indicated atmospheres; harvest II, 2 August; harvest III, 9 August; harvest IV, 15 August.)¹

The similarity in the results of the experiments on symbiotic N fixation to that due to *Azotobacter* suggests that the mechanisms are similar if not identical. In both cases the rate of reaction is independent of pN_2 till the latter is reduced to about 0.15 atm. and the Michaelis constants are of the same order. Both types of fixation are inhibited by hydrogen and the inhibition is reversible and competitive. The K values for N_2 and H_2 respectively are 0.01 and 0.08 for *Azotobacter* and 0.05 and 0.14 for the symbiotic (red clover) system; that is, the ratio of the affinity of the N_2 and H_2 systems is 8 in the *Azotobacter* and 3 in red clover.

Further light has been thrown on the mechanism by the use of the stable isotope N^{15} . It will be recalled that very early Winogradsky noticed that nitrogen fixation with *Cl. pastorianum* was inhibited by ammonium salts. When *Azotobacter* rapidly fixing N_2 was given combined N in the form of 10 p.p.m. ammonium salt labelled with 32 atoms excess of N^{15} , samples withdrawn 1 minute

¹ Wilson, Umbreit and Lee, *Biochem. J.*, **32**, 2087 (1938).

after the addition already showed a detectable amount of N^{15} in the cells; the uptake was linear for 20 minutes, after which the ammonium salt became exhausted. Analysis of the N^{15} in the cells showed that the whole of the growth after the addition of the salt was due to the ammonium ion, i.e. that the cell had switched over immediately and completely to ammonia utilisation. This indicates that the machinery for ammonia utilisation exists ready for use in the cells fixing molecular nitrogen and the cells turn over from N fixation to NH_3 utilisation with no period of adaptation.

When nitrate N 10 p.p.m. with 32 atom% excess N^{15} was used instead of ammonium N, a considerable period of adaptation occurred before nitrate N was taken up by the cell, the nitrate N taken up in 30 minutes being equal to the ammonium N taken up in 1 minute. The most probable interpretation of these results is that nitrate is not absorbed as such but is first reduced to ammonia. This was proved as follows: to determine the relative utilisation of nitrate and ammonia N, ammonium nitrate with only the ammonium ion enriched with N^{15} was supplied to cultures in an atmosphere of hydrogen which prevented N fixation. Inoculum was taken from a culture adapted to ammonium nitrate and the medium contained 100 p.p.m. each of NO_3^- -nitrogen and NH_4^+ -nitrogen, the latter enriched with 32 atom% excess N^{15} . Cultures were harvested at intervals and analysed as shown in Table 9. The last two columns in Table 9 were calculated from the atom% N^{15} excess in the cells and from the initial 28.90 atom% of N^{15} excess in the NH_4^+ -nitrogen and the 0.00N N^{15} excess in the NO_3^- -nitrogen of the medium. Thus at 8 hours

$$\frac{19.62 \times 100}{28.9} = 67.9\%$$

of the cellular nitrogen was derived from NH_4^+ and the remainder from NO_3^- . Thus although the culture was adapted to NO_3^- it used NH_4^+ preferentially; as the NH_4^+ became exhausted both were used at about the same rate. The same experiment shows that NO_3^- was reduced to NH_4^+ . At 8 hours the NH_4^+ -nitrogen used was 0.77 mg. and the NO_3^- -nitrogen disappeared was 2.31 mg., whereas the isotope data showed that two-thirds of the N assimilated came from NH_4^+ . This shows that the NH_4^+ of the medium was being added to by the reduction of nitrate, and is corroborated by the falling percentage of N^{15} in the NH_4^+ of the medium in the course of the experiment due to its dilution with NH_4^+ from reduction of nitrate (column 5). Furthermore, to attain the N^{15} level in the cells observed at 8 hours, 1.98 mg. NH_4^+ -nitrogen would be required whereas only 0.77 mg. was shown to have disappeared by the isotope analysis. This was calculated on the supposition that no dilution had occurred, and indicated the percentage of cellular

N arising from the original NH_4^+ (with 28.9 atom % N^{15}) but not from NH_4^+ derived from nitrate. Further experiments showed that both unadapted cultures and those adapted to nitrate used NH_4^+ in preference to NO_3^- , and growth on the latter occurred only after 1-2 hours. These experiments are in favour of the view that NH_3 is the first stable intermediate formed in fixation by *Azotobacter*.¹

TABLE 9

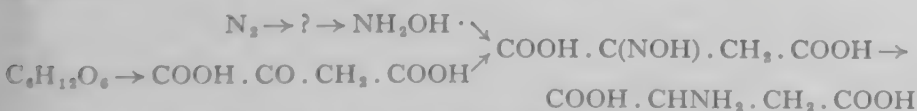
UPTAKE OF AMMONIA AND NITRATE-N BY *Azotobacter vinelandii*

Time after inoculation	Total N in cells	Atom % N^{15} excess in cells	NH_4^+ -N in medium	Atom % N^{15} excess in medium	NO_3^- -N in medium	% cell-N from original NH_4^+	% cell-N from NO_3^-
0	—	—	7.46	28.90	7.80	—	—
8	2.92	19.62	6.69	24.94	5.49	67.9	32.1
14	9.04	16.67	3.29	13.63	3.08	57.7	42.3
20	13.61	13.91	1.31	9.65	0.62	48.2	51.8
24	14.43	13.48	0.85	7.69	0.26	46.6	53.4

In order to get indications of the further route followed by the nitrogen fixed, a culture of *A. vinelandii* growing on nitrogen was given nitrogen enriched with N^{15} for 90 minutes. The cells were then harvested, hydrolysed and fractionated. The highest level of N^{15} was found in the glutamic acid and the second highest in the aspartic acid fraction. These findings indicate that the ammonia formed by fixation enters the cell via the 4- and 5-carbon dicarboxylic acids. Confirmation of this was obtained by using ammonium salts enriched with N^{15} in place of nitrogen. The distribution of N^{15} was again found almost exclusively in the glutamic and aspartic fractions; thus the hypothesis that N_2 and NH_3 enter the cell by the same route is supported.^{2, 3}

The hydroxylamine hypothesis

This scheme for the mechanism of nitrogen fixation by the symbionts was put forward by Virtanen⁴ and is expressed by the scheme



The evidence adduced for this scheme is briefly as follows:

1. Cultures of legumes in sterile sand with the appropriate *Rhizobia* on nitrogen-free medium excrete considerable amounts

¹ Burris & Wilson, 1946.

² Burris, 1942.

³ Burris & Wilson, 1944.

⁴ Virtanen, 1938.

of nitrogenous products consisting almost entirely of *L*-(+)-aspartic acid and β -alanine; in addition 1-2% of the excreted nitrogen was found as the oxime of oxalacetic acid ($\text{COOH} \cdot \text{C}(\text{NOH})\text{CH}_2 \cdot \text{COOH}$).¹

2. This was corroborated by the observation that hydroxylamine condenses more easily with oxalacetic than with pyruvic acid and that seedlings can absorb the oxime of oxalacetic acid at low concentrations but do not absorb the oxime of pyruvic acid (Table 10).

TABLE 10

1 litre medium 1.3 kg. dry sand. 1 pea in each flask
Period, 21 Feb.-30 March

Oxime of oxalacetic supplied, mg. N	Plant	
	Dry wt., g.	N. mg.
0	0.370	6.9
1	0.454	7.7
3	0.603	10.6
5	0.539	11.1
15	0.396	8.7
30	0.291	6.7
60	0.365	6.4
Oxime of pyruvic acid supplied, mg. N		
1	0.361	6.3
5	0.375	5.9
10	0.337	5.7
30	0.354	5.2
60	0.360	7.1

3. Nodules detached from the root either whole or crushed fix nitrogen in the presence of oxalacetic, but not in its absence, nor when it is replaced by glucose. This shows that the oxalacetic is supplied by the plant and that fixation occurs in the nodules. The bacteria apart from the nodules, even in the presence of oxalacetic acid, do not fix nitrogen.

50 g. of nodule was removed from the root and divided into five lots of 10 g. and placed in five flasks with sterile sand and nitrogen-free medium; 20 mg. of oxalacetic acid was added to two flasks; the three other flasks served as controls, two at the beginning and one at the end of the experiments (see Table 11). When this fixation, 6-7 mg./10 g. nodule in 24 hours, is compared with that occurring in the root nodule in the growing plant, it is found to be of the same order.

¹ Virtanen & Laine, 1938 (2).

² Ibid., 1939.

TABLE 11

Control start (1)	69.9
" " (2)	69.1
" after 24 hr.	70.1
Exp. with oxalacetate after 24 hr. (1)	75.0
" " " (2)	78.4

Against this scheme must be set the following facts:

1. Workers in other departments have failed to corroborate the large excretion of $I(+)$ aspartic acid and β -alanine.¹
2. The oxalacetic acid found in leguminous plants first reported was some ten times too high, the new values found amounting to 24-94 μ g. oxalacetic acid per g. fresh weight of legume tissue, α -ketoglutaric acid being present in comparable amounts.²
3. The fixation of nitrogen by excised nodules in the presence of oxalacetic acid has not been completely confirmed.³

At present the balance of evidence is in favour of the fixation of nitrogen via ammonia, but until more is known about the mechanism the possible role of hydroxylamine cannot be excluded. The powerful tool provided by isotopic nitrogen is now available and we may look forward to a solution of the problem by its use.

Meanwhile the curious connection between nitrogenase (the enzyme or enzymes catalysing the fixation process) and hydrogenase is interesting. The inhibition of fixation by hydrogen is true of both free and symbiotic fixation, and for fixation by *Nostoc muscorum*,⁴ but the presence of hydrogenase, the only enzyme so far reported as activating molecular hydrogen, has been confirmed only in the case of *Azotobacter*;⁵ the reported presence of this enzyme either in nodule bacteria or in the nodules not having been substantiated.⁶

There seems to be some relation between hydrogenase and nitrogenase. Nitrogen fixation in all three types is inhibited by carbon monoxide in low concentration (0.0001 to 0.0005 atm. in the case of red clover and ten times as high for *Azotobacter*); the inhibition is non-competitive.^{7, 8} The oxidation of hydrogen by oxygen is inhibited by cyanide and carbon monoxide; this may of course merely be an effect on cytochrome oxidase,⁹ and it will be remembered that it has been shown that hydrogenase is an iron-containing enzyme.¹⁰

It has been shown that conditions which inhibit nitrogen fixation with *Azotobacter* also decrease or inhibit hydrogenase activity; such, for example, are the presence of low concentrations of NH_4^+

¹ Burris & Wilson, 1945, from which see further references.

² Vermanen *et al.*, 1943. ³ Allison *et al.*, 1942. ⁴ Burris & Wilson, 1945.

⁵ Wilson & Wilson, 1942. ⁶ Wilson *et al.*, 1943 (2). ⁷ Lind & Wilson, 1941.

⁸ Wilson & Lind, 1943.

⁹ Wilson & Wilson, 1943.

¹⁰ Lee & Wilson, 1943.

in both adapted and unadapted cells and the presence of NO_3^- in adapted cells. When nitrogen fixation is inhibited owing to growth in H_2 and O_2 , hydrogenase is also reduced. Sources of combined nitrogen which cause little or no inhibition of nitrogen fixation do not inhibit hydrogenase formation; the presence of hydrogen during growth, which might be expected to increase hydrogenase, actually inhibits it.¹ It would, however, be rash at this stage to draw conclusions from the parallelism between the formation of hydrogenase and nitrogen-fixing power in view of the fact that hydrogenase has not been demonstrated in *Rhizobia* or in nodules.²

A striking discovery in connection with symbiotic nitrogen fixation is that of the formation of a hæmoglobin in the root nodules of legumes.³ This has been extracted and purified to the extent of 40–50% by crushing fresh nodules of the soya bean in saturated ammonium sulphate and azide in order to prevent the formation of quinonic substances and consequent darkening of the extract. Sodium hyposulphite was added to keep the iron of the pigment reduced. The preparation has two bands, α at 574 $\text{m}\mu$ and β at 540 $\text{m}\mu$. On reduction with sodium hyposulphite both bands disappear and are replaced by one band at 557 $\text{m}\mu$. This change can also occur without a reducing agent by warming the solution to 37° and evacuating on a water-pump; on admitting air the two-banded spectrum reappears. This shows that the two bands belong to an oxygenated and not to an oxidised pigment, and that the combination of the pigment with oxygen is reversible. On treating the oxygenated compound with carbon monoxide its two bands are replaced by two more diffuse bands at 564 $\text{m}\mu$ and 538 $\text{m}\mu$; and treatment of the oxygenated compound with potassium ferricyanide leads to a change from red to brown and the two absorption bands are replaced by the absorption spectrum of methæmoglobin. At 15° this hæmoglobin is 50% dissociated at an oxygen pressure of 0.1 mm.; the relative affinity of the pigment for O_2 and CO at 15° as expressed by the equation

$$K = \frac{(\text{HbCO})(\text{PO}_2)}{(\text{HbO}_2)(\text{PCO})} \text{ is about } 37.$$

Exactly what the function of this hæmoglobin is in nitrogen fixation is unknown; it has been found in the actively fixing nodules of every legume so far examined and may account for the inhibition of fixation in legumes by very low pressures of CO. This hæmoglobin seems to be the result of the joint action of the bacteria and the plant tissue, since neither alone contains any.

¹ Lee & Wilson, 1943.

³ Keilin & Wang, 1945.

² Wilson, Burris & Coffee, 1943.

CHAPTER IX

AUTOTROPHIC BACTERIA

THE NITRIFIERS

Discovery of the biological origin of soil nitrification

When, in 1862, Pasteur demonstrated that the oxidation of alcohol to acetic acid was the work of a micro-organism, the idea that the oxidation of ammonia to nitrate, which had long been known to occur in the soil, might have a similar origin suggested itself to him; he himself, however, did not follow up the subject, and it was not until fifteen years later that its truth was demonstrated.

In 1877 Schloesing and Muntz,¹ in a few very simple experiments, demonstrated quite conclusively the biological origin of the nitrification process. They used for this purpose a tube 1 metre long, filled with sand and chalk previously sterilised by incineration; sewage water was poured daily into the upper end of the tube. For twenty days the ammonia content of the effluent remained constant and no nitrate could be detected; then suddenly nitrate made its appearance and the ammonia content fell to nothing. The nitrification, once started, continued for four months. It then occurred to the investigators to try the effect of an antiseptic on the reaction and a current of chloroform was drawn through the tube; after ten days the nitrate of the effluent had completely given place to ammonia. The fact that the liquid took eight days to traverse the length of the tube accounted for the lapse of time between the addition of the antiseptic and the complete disappearance of nitrate from the effluent. In order to restore the nitrification some washings from garden soil were poured through the tube; nine days later nitrate reappeared in the effluent. The authors also showed that nitrification could be stopped by heating the tube to 100° and again restarted by the addition of soil washings.² They also attempted to induce the oxidation of ammonia by the inoculation of various organisms characterised by their high oxidising powers on organic substrates (*P. glaucum*, *A. niger*, *Mycoderma vini*, *Mycoderma aceti*, etc.);³ this met with no success. Finally, they established among the conditions favouring nitrification a slightly alkaline reaction and

¹ Schloesing & Muntz, 1877 (1), (2).

² Ibid.

³ Ibid., 1878

a free access of oxygen ; the appearance of nitrites in the effluent was also noted.¹

The isolation of the nitrifying organisms

The work of Schloesing and Muntz made it almost certain that the oxidation of ammonia to nitrate in the soil was due to microbes. All that remained to clinch the matter was to isolate the organisms responsible and demonstrate their action apart from the soil. The recent introduction of "solid media," i.e. nutrient gelatin plates, had rendered comparatively easy the work of isolating pure strains of bacteria free from contaminating organisms, and seemed to give every promise that the active agents of soil nitrification would not long lie hidden. But, as often happens in the history of science, the last lap was unexpectedly difficult, and indeed took thirteen years to traverse. Many investigators experienced the same disappointment. Nitrification in soil suspensions was easily achieved ; the cultures were then plated on to gelatin plates, subcultures were made from individual colonies, and their nitrifying power tested on solutions containing ammonium salts. Negative results were invariably obtained ; some false hopes were indeed raised by the occasional appearance of nitrites in the culture media, but these were subsequently shown to have arisen through the reduction of nitrates, and to be no indication of the progress of nitrification. In 1879 Warington,² working at Rothamsted, noted the inhibiting effect of glucose on the production of nitrates by soil cultures, and the improved, but still uncertain, results obtained when the organic food consisted only of tartrates ; he also observed the necessity for carbonates and the occasional replacement of nitrites for nitrates.³ In fact, Warington had already to his hand all the data which, some ten years later, enabled Winogradsky to unravel the problem.

The repeated failure of numerous investigators to regain from the surface of nutrient gelatin the nitrifying organisms which were undoubtedly present in the soil culture from which the plates were sown, at length convinced Winogradsky that the gelatin plate method which had proved so successful for the isolation of disease germs must be unsuited to the present purpose : "Chaque methode, quelque excellente qu'elle soit dans un cas, peut refuser le service dans un autre."⁴

His own work on the sulphur and iron bacteria also suggested to him the possibility that organisms adapted to utilise the energy liberated by the oxidation of ammonia might be ill-adapted to form colonies on nutrient gelatin, and so elude the pursuit of

¹ Schloesing & Muntz, 1879.

³ Ibid., 1884, 1888.

² Warington, 1879.

⁴ Winogradsky, 1890.

bacteriologists employing this medium. He therefore tried a simple medium consisting of potassium phosphate, magnesium sulphate, potassium carbonate, ammonium chloride with 0.1% potassium tartrate as the sole source of carbon. Actively nitrifying soil was sown into this solution, but the result showed hardly any nitrification. Each item of the medium was then omitted in turn, with no result, until finally the organic matter was left out. The result was immediate and intense nitrification. The nitrifying organisms having thus been obtained apart from the soil, their isolation in pure culture was the next step. Gelatin proved to be useless, since nothing that would nitrify would grow on gelatin. Finally, by repeated subcultures into inorganic media, a culture was obtained which gave no growth on nutrient gelatin and which, for the time being, was thought to be pure. Subsequently Winogradsky¹ employed a solid medium in which the appropriate salts in solution were solidified by silicic acid. On this so-called "silica jelly" (Medium III) colonies of nitrifying organisms alone developed, and could easily be obtained free from other bacteria.

The chemical processes of nitrification

The next stage in the problem was to elucidate the steps by which the ammonia was oxidised to nitrate. Schloesing and Muntz and also Warington had observed that nitrification often took place in two stages, i.e. (1) ammonia to nitrite, (2) nitrite to nitrate, whilst sometimes it appeared to occur in one stage. Meanwhile it had been suggested by Duclaux that two separate organisms might be severally responsible for the two reactions.

Winogradsky started investigations on numerous samples of soil from different parts of the world, using a medium (No. I) containing the usual salts with 0.2% ammonium sulphate and no organic carbon.

He followed the disappearance of ammonia by means of Nessler's reagent, the appearance of nitrite by the starch iodide test, and the appearance of nitrate by the *m*-phenylene diamine test subsequent to the removal of nitrite.² The result of a typical experiment is given in Table 1.

From a number of results closely resembling the one quoted, it was shown that the oxidation of ammonia to nitrite always preceded that of nitrite to nitrate, and that as a rule the latter did not begin whilst any ammonia remained unoxidised. On repeated subcultivation, the power of the daughter cultures to effect the oxidation of nitrite to nitrate was found to fall off and finally disappear. Such cultures never regained the lost power,

¹ Winogradsky, 1891 (1), (2).

² Ibid. (2).

TABLE I

Day	Presence of		
	Ammonia	Nitrite	Nitrate
I	+ + +	o	—
11	o	+ + +	—
18	o	+ + +	—
21	o	+ +	—
26	o	+	+ + +
32	o	o	+ + +

even when subcultivated on to silica jelly or reinoculated with sterile earth. Winogradsky attributed this loss to subcultivation in media in which nitrites were for a time absent, and showed that no loss took place if the media used (like the soil) contained nitrites from the beginning. From these results Winogradsky was convinced that two organisms were concerned in nitrification: by repeated subcultivation into media containing (1) ammonia but no nitrite, (2) nitrite but no ammonia, and subsequent plating on to silica jelly, two types of organisms could be separated from every nitrifying soil, the one effecting the oxidation of ammonia, the other that of nitrite.¹

Though these differed morphologically according to the part of the world from which they came, all were alike in their chemical behaviour and in requiring the same conditions, viz. the presence of an ammonium salt or nitrite to oxidise, strongly aerobic conditions, and the presence of carbonate—with the usual salts—in the medium.

The task of obtaining the nitrifiers in pure culture has presented difficulties to many workers ever since the early work of Winogradsky. The technique has been reinvestigated by Kingma-Boltjes,² and his work should be studied by anyone attempting the problem. He showed the necessity for calcium in the development of *Nitrosomonas* in a minimum concentration of 1 mg./l. and confirmed the observation of Fred and Davenport³ that "Nährstoff-Heyden" (egg albumin submitted to a short-term acid hydrolysis) in amounts up to 0.75% assists the growth of both types of nitrifier.

Energy relations

The chemical behaviour of this group of bacteria is peculiarly interesting from the point of view of metabolism. It was originally shown by Winogradsky,⁴ and later confirmed by Meyerhof⁵ and

¹ Winogradsky, 1890.

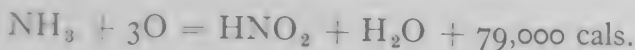
³ Fred & Davenport, 1921.

⁵ Meyerhof, 1916 (1).

² Kingma-Boltjes, 1935.

⁴ Winogradsky, 1890.

many others, that these organisms are able to develop in media from which all trace of organic compounds has been rigorously excluded. The energy necessary for synthesis must, therefore, be obtained exclusively from the oxidation of ammonia or of nitrite; thus the nitrite producer (*Nitrosomonas*) utilises the energy liberated in the reaction



whilst the nitrate producer (*Nitrobacter*) depends on the reaction



Winogradsky determined the ratio of nitrogen oxidised to carbon entering into synthesis in the two cases and obtained the

result $\frac{\text{N}}{\text{C}} = 35$ for *Nitrosomonas*, 135 for *Nitrobacter*. Later,

Meyerhof, working in more favourable conditions, obtained the

value $\frac{\text{N}}{\text{C}} = 101$ for *Nitrobacter*. As would be expected, the values

obtained for these ratios are roughly inversely proportional to the amount of heat liberated by the oxidation of 1 g. of nitrogen in the two cases:

$$\frac{35}{101} = 0.346 \qquad \frac{21,600}{79,000} = 0.276$$

From these ratios the energy efficiency can be calculated and compared with that of organisms employing organic compounds as sources of energy. The heat of combustion of *Nitrobacter* cells has not been determined, and in the absence of this figure the ratio: heat of combustion of cells/heat lost by the medium in the production of the cells, can only be approximate. The amount of carbon synthesised, however, having been determined, this was assumed for purposes of calculation to possess the same heat of combustion as in glucose, i.e. 113,000 cal. per g. atom.

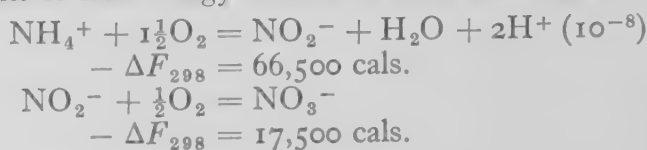
From these experimental data Meyerhof found that the oxidation of 1 g. mol. of KNO_2 results in the assimilation of $\frac{14}{12 \times 101}$ g. atoms of carbon, of which the heat of combustion

$$= \frac{14 \times 113,000}{12 \times 101} = 980 \text{ cal.}$$

but since 1 g. mol. of KNO_2 , on oxidation to KNO_3 , liberates 21,600 cal., the total energy efficiency of the process

$$= \frac{980 \times 100}{21,600} \% = 4.53\%$$

Baas-Becking and Parks¹ have calculated the relation from the standpoint of free energy instead of from heats of combustion:



The changes are calculated from concentrations of ammonium and nitrite shown by Meyerhof to be optimal for the organisms, viz.

$$(\text{NH}_4^+) = 0.005 \text{ M}, \text{ H}^+ = 10^{-8}, (\text{NO}_2^-) = 3.03 \text{ M}$$

The reduction of 1 g. mol. CO_2 to glucose requires 118,000 cal. free energy; for *Nitrobacter* the free energy efficiency

$$= \frac{14 \times 118,000 \times 100}{12 \times 101 \times 17,500} = 7.8\%$$

$$\text{For } \textit{Nitrosomonas} = \frac{14 \times 118,000 \times 100}{12 \times 35 \times 66,500} = 5.9\%$$

From these figures one would expect about 95% of the energy liberated by the oxidation of nitrate to appear as heat. Meyerhof obtained figures for *Nitrobacter* roughly in accordance with this expectation. (Table 2.)

TABLE 2
HEAT EVOLVED FROM THE OXIDATION OF NITRITE

	A Measured in calorimeter, g. cal.	B Calculated from nitrate found, g. cal.	$\frac{(B-A) \times 100}{B}$
1	31.6	32.4	2.5
2	30.0	31.8	6.0
3	29.1	29.8	2.5
4	23.5	25.5	9.0

Conditions affecting nitrification

Certain of the conditions governing the growth of the nitrifying bacteria were noted by Winogradsky and his school—such, for example, as the necessity for carbonates of calcium or magnesium, the inhibitive effect of many organic compounds and of ammonium salts. The subject was later amplified by Meyerhof in a detailed study of both types of bacteria.² He considered that the action of carbonates was due to their buffering effect, and demonstrated their necessity by showing the extreme sensitiveness of the organisms to hydrogen-ion concentration. This is apparent from

¹ Baas-Becking & Parks, 1927.

² Meyerhof, 1916 (1), (2), and 1917.

his experimental curves (Figs. 1 and 2), in which rate of oxidation is plotted against pH.

The optimum pH for the oxidation of ammonia lies between 8.5 and 8.8, that for nitrite oxidation between 8.3 and 9.3. The

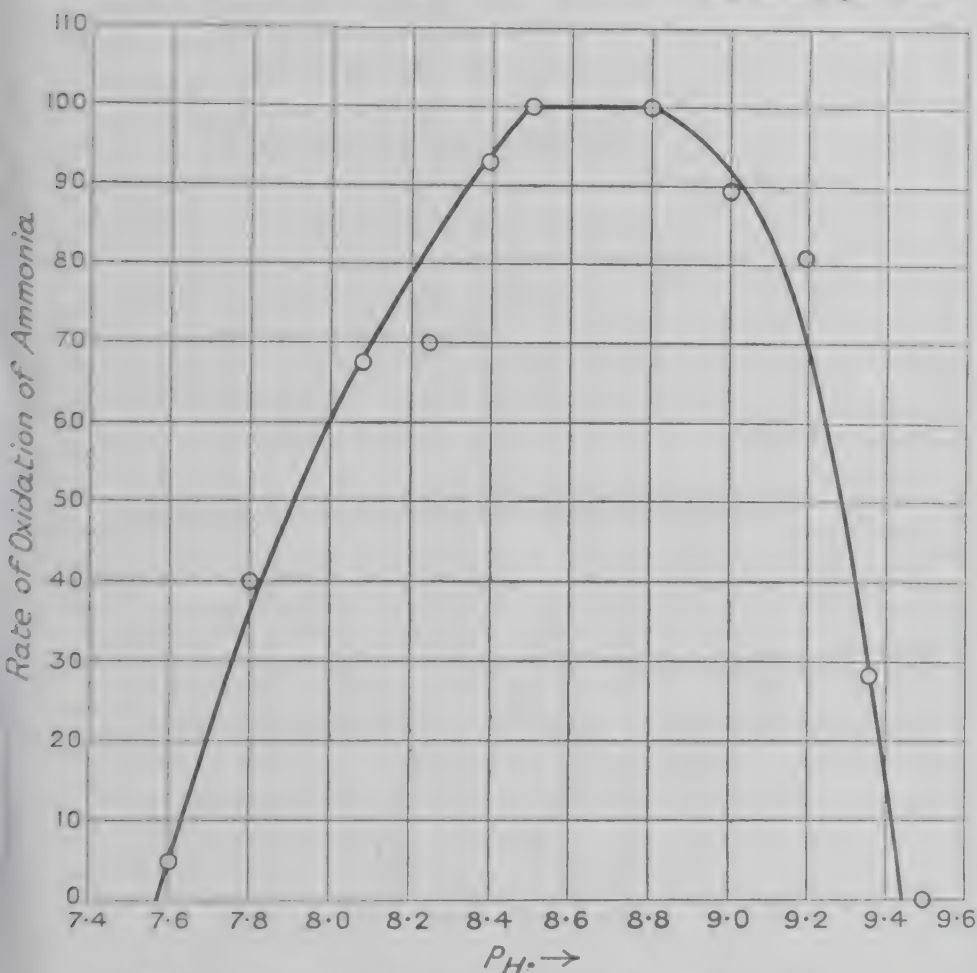


FIG. 1.—Rate of oxidation of ammonia by *Nitrosomonas*¹

pH of a 0.2 molar solution of sodium bicarbonate is 9.15; the simultaneous presence of sodium carbonate and free access of carbon dioxide is seen therefore to provide the conditions best suited to maintain the reaction most favourable to the development of these bacteria. It is important from the standpoint of soil economy to note that organisms isolated from peat soils (pH 4.6) display a greater tolerance for acid, nitrification continuing as low as pH 4.1.²

The high optimum alkalinity and the rapid falling off in activity

¹ Meyerhof, *Pflügers Arch.*, 1917, 166, 255.

² Meek & Lipman, 1922.

on both sides of this suggest that secondary inhibitory effects are becoming operative both on the acid and alkaline sides. Probably, as Meyerhof suggested, the rapid drop on the alkaline side is due to the penetration of the cell by free ammonia. This is supported

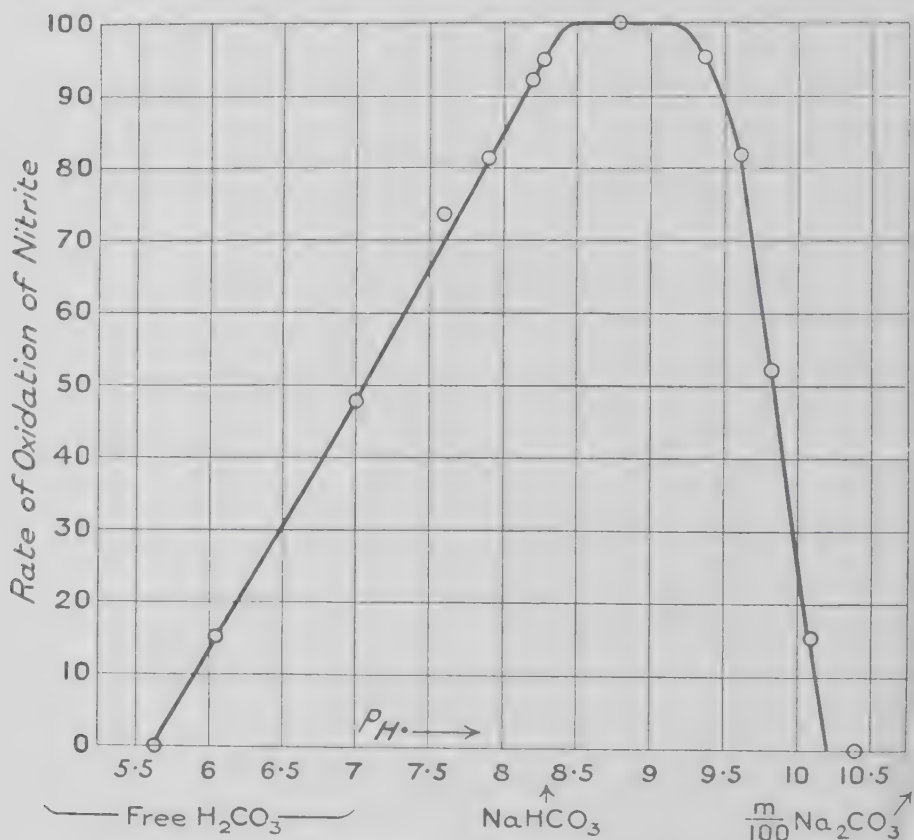


FIG. 2.—Rate of oxidation of nitrite by *Nitrobacter*¹

by the form of the curve obtained when the rate of oxidation of ammonia is plotted against concentration of ammonium salts (Fig. 3); this shows a rapid drop in rate of oxidation after the comparatively low optimum concentration of ammonium salts has been reached.

It was shown both by Warrington and by Winogradsky and confirmed by Meyerhof that the oxidation of nitrite also is unfavourably affected by ammonium salts; that this also is an effect of free ammonia is suggested by the fact that a given concentration of ammonium salt has a more powerful inhibitory action at a high pH than at a low one. This is made clear by the following figures obtained by Meyerhof (Table 3).

¹ Meyerhof, *Pflügers Arch.*, 1916, **164**, 416.

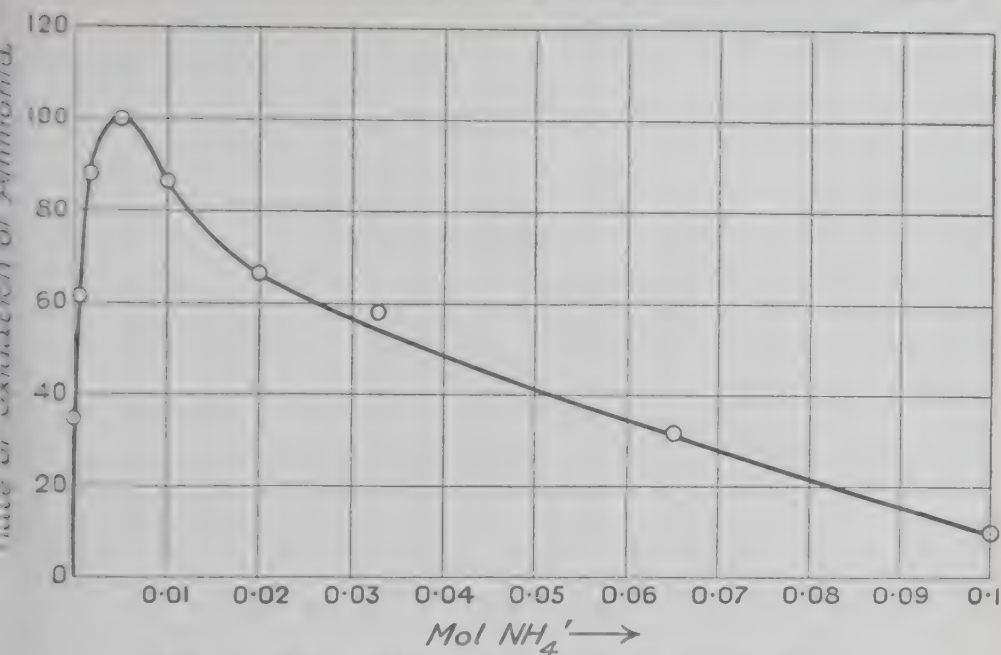
FIG. 3.—Rate of oxidation of ammonia by *Nitrosomonas*¹

TABLE 3

EFFECT OF AMMONIUM SALTS ON THE OXIDATION OF NITRITE

pH	Concentration of (NH_4) $_2$ SO $_4$	Inhibition of the oxidation of nitrite
7.8	$\frac{N}{1000}$	8%
9.4	$\frac{N}{1000}$	55%
7.6	$\frac{N}{500}$	30%
9.5	$\frac{N}{500}$	77%

The curve obtained by Meyerhof showing the influence of nitrite concentration on the production of nitrate (Fig. 4) is similar in character to that showing the effect of ammonium salts on nitrite production. The form of this curve also may be explained by the consideration that nitrite acts as a cell poison, possibly by reacting with the free amino groups of the cell proteins.

The peculiar behaviour of nitrifying bacteria towards carbon compounds has already been referred to. Winogradsky and Omeliansky² showed that not only were these bacteria able to develop in media from which organic compounds were rigidly

¹ Meyerhof, *Pflügers Arch.*, 1917, 166, 245.² Winogradsky & Omeliansky, 1899.

excluded, but that many organic compounds actually exert an inhibitory effect on the oxidation. They further showed that this inhibition is due to the non-development of the bacteria

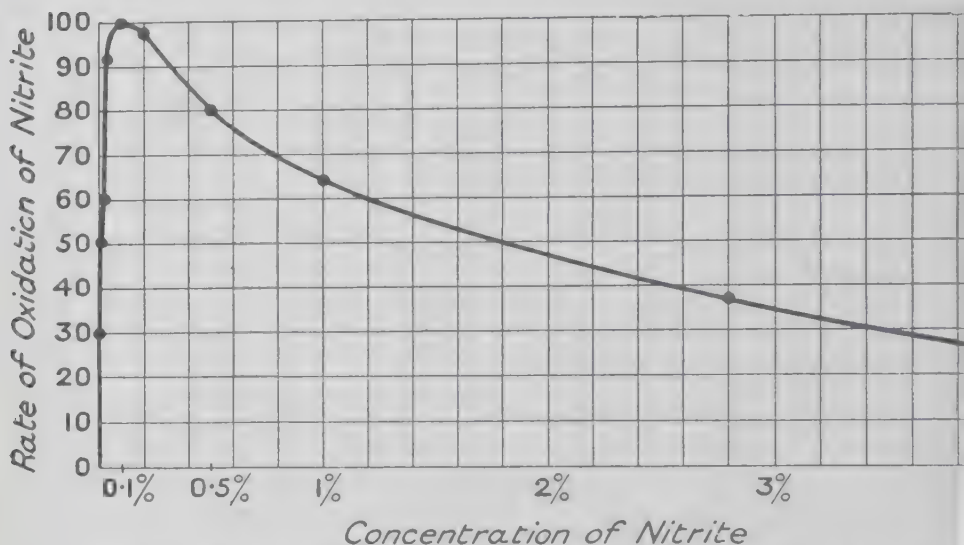


FIG. 4.—Effect of concentration of NaNO_2 on rate of oxidation of nitrite to nitrate¹

inoculated, the cultures, after a period of incubation, being completely sterile. Table 4, compiled by Meyerhof from the figures

TABLE 4²

EFFECT OF SOME ORGANIC COMPOUNDS ON THE GROWTH OF AND OXIDATION BY *Nitrobacter*

Compound	Concentration	Effect on growth	Concentration	Effect on oxidation
Glucose	0.0025 M. 0.015 M.	Delayed Completely inhibited	0.03 M. 0.6 M.	Not affected 10% inhibition
Asparagine	0.003 M. 0.05 M.	Delayed Completely inhibited	0.8 M. 0.15 M.	25–30% inhibition 10–20% inhibition
Urea	0.15 M. 0.3 M.	Delayed Completely inhibited	0.15 M. 0.5 M.	No inhibition 32% inhibition

of Winogradsky and Omeliansky, illustrates the effect of some carbon compounds on (a) growth, (b) oxidation by cells already formed.

The toxicity of glucose is less in the presence of sand or soil than in liquid media. Thus oxidation by *Nitrobacter* was accelerated by glucose in concentrations up to 0.2%, above

¹ Meyerhof, *Pflügers Arch.*, 1916, 164, 390.

² *Ibid.*, 1916.

which inhibition occurred.¹ With *Nitrosomonas* 0.02% of glucose acts beneficially in sand culture, whilst in liquid culture the same concentration is partially inhibitive.

More recently Kingma-Boltjes² showed that pure cultures of both types of nitrifier develop larger colonies if 0.7% "Nährstoff-Heyden" is added to the inorganic medium. This preparation appears to be an incomplete acid digest of egg albumin. In liquid medium it also acts favourably but does not replace any constituent of the inorganic medium, the organisms continuing their autotrophic mode of life. In fact the only evidence that the substance is used by the organism is that the colonies are larger in its presence than its absence and the possibility is not excluded that its action is physical rather than chemical. Various peptones and also glycine exert a definitely toxic effect both on growth and respiration, toxicity being roughly proportional to the amino groups. The same investigator showed that the respiration of both nitrifiers had a much higher tolerance for glucose than was found by Winogradsky and Meyerhof, only slight inhibition being caused by 4.0% glucose. Growth of both nitrifiers also occurred in media containing the same high concentration of sugar, which itself was not attacked.

The nitrifying organisms in common with many other autotrophic bacteria display a strict specificity towards the material which they oxidise; thus not only is *Nitrobacter* inactive towards ammonia and *Nitrosomonas* towards nitrite, but neither is able to utilise sulphite or phosphite or any carbon compound as source of energy.

Winogradsky has attached much importance to the view that inability to multiply in organic media is a necessary corollary to the autotrophic nature of these nitrifying organisms, and has reiterated that failure to reproduce when subcultivated into meat broth is to be regarded as an essential criterion of the purity of the culture of both types of nitrifiers. This view has been challenged more than once. Burri and Stutzer,³ for example, reported that they had isolated by means of silica jelly a soil organism resembling *Nitrobacter*, but able to develop in broth, though on this medium it rapidly lost its nitrifying power, which it failed to regain when subcultivated back into nitrite media. Winogradsky⁴ immediately sent for the culture and was able to demonstrate by using nitrite medium with agar instead of with silica jelly that three contaminating heterotrophic organisms were present along with the true *Nitrobacter* and that these grew when subcultivated into broth, though when returned to nitrite media

¹ Coleman, 1908.

² Burri & Stutzer, 1895.

³ Kingma-Boltjes, 1935.

⁴ Winogradsky, 1896.

they naturally failed to oxidise it. Much later Beijerinck¹ made a report similar to that of Burri and Stutzer; this, owing to the condition of Europe at the time of publication, failed to attract Winogradsky's attention until 1922,² when he expressed the opinion that the organism was not in pure culture.

Sack³ reported the isolation from soil of an ammonia oxidiser which he could grow either autotrophically or in broth or peptone. In both conditions it oxidised ammonia, though not nitrite. Anaerobically it reduced nitrate to nitrite, but did not further attack nitrite. Glucose, fructose, sucrose, maltose and asparagine could serve as sources of carbon, and ammonia could be oxidised as well in their presence as in their absence. He also described four strains of *Nitrobacter*, one of which oxidised ammonia as well as nitrite; the oxidations were inhibited by organic compounds. Proteins were decomposed with production of ammonia and anaerobically nitrate was reduced to nitrite.

The subject has again been brought under review by Winogradsky⁴ himself and by Kingma-Boltjes.⁵ Numerous instances reported in the literature of nitrification occurring in organisms growing heterotrophically have been examined, and serious objections to the technique employed have been made in every case. Certain common sources of error have been pointed out; these are as follows. Heterotrophic contaminants continue to grow alongside the autotrophic nitrifiers even after repeated subculture in inorganic medium; *Hyphomicrobium vulgare* (Stutzer) is a case in point.⁶ Such organisms frequently form colonies on silicic jelly which may be mixed with autotrophants (especially *Nitrosomonas*). On subcultivating such a mixed colony into broth media the heterotrophant grows and produces ammonia. If the concentration of amino-acids be not too high the autotrophant may also grow weakly and produce nitrite which is attributed to the heterotrophant. Alternatively nitrite may appear in media due to gas fumes and give a weak reaction with the Griess-Ilosvay reagent which is falsely attributed to nitrification. The Griess-Ilosvay reagent gives a positive colour test with 0.1 μ g. nitrite-N/ml. Vigorously nitrifying cultures produce 0.2 mg. (200 μ g. nitrite-N/ml. in four days and less than one-tenth that amount should be disregarded. Also nitrite and nitrate formed as a result of nitrification must correspond with the ammonia and nitrite respectively which disappear. It must also be remembered that ammonia disappears spontaneously at 30° from solutions with magnesium carbonate owing to high pH (9.1) and this must be duly checked.

¹ Beijerinck, 1914.

⁴ Winogradsky, 1933.

² Winogradsky, 1922.

⁵ Kingma-Boltjes, 1935.

³ Sack, 1925.

⁶ Ibid.

The subject of the toxicity of organic compounds for *Nitrobacter* has also been studied by Fred and Davenport.¹ It was shown that of various organic media meat broth was the most definitely toxic, but that when diluted to half its normal strength it was practically non-toxic. The toxic material was non-volatile, and could be extracted by alcohol or ether, and the residue was then non-toxic; 1% solutions of gelatin, peptone, casein, milk and yeast water were non-toxic, i.e. the organisms could be subcultivated after two to six weeks; 0.1% concentrations of peptone, skimmed milk, beef extract and beef infusion, when added to a nitrite medium, favoured growth; on the other hand, the same concentrations of gelatin, urea and asparagine retarded it.

The influences inhibiting oxidations by the nitrifying organisms have received an extended study by Meyerhof with a view to comparing this form of "respiration" with that of the oxidation of organic compounds by atmospheric oxygen. Inhibition of *Nitrobacter* by narcotics (e.g. the substituted urethanes and amyl alcohol) differs from that of tissues, red blood cells, liver, etc., previously studied, in that the inhibition in the latter case is approximately proportional to the concentration of the narcotic, whilst in the case of *Nitrobacter* the amount of inhibition is proportionately greater as the concentration rises. For example, 42 millimol. isobutylurethane inhibited nitrate production by 10%, 84 millimol. by 96%; thus when inhibition (ordinates) is plotted against concentration of narcotic (abscissæ) the curve is convex in respect of the base. Furthermore, when the inhibition of two narcotics was determined separately and afterwards together, the effect produced was more than the sum of each separately. For example, 42 millimol. isobutylurethane caused an inhibition of nitrate production amounting to 10%; that of 100 millimol. propylurethane 23%; the two together an inhibition of 100%.

When inhibition by cyanide and narcotics was combined, the total inhibition was less than by either separately. For example, 5×10^{-6} M. KCN caused an inhibition of 69%, 42×10^{-3} M. isobutylurethane an inhibition of 5%; the combined effects amounted to an inhibition of 73%. In low concentrations of narcotic the combined effects of narcotic and cyanide were lower than that of cyanide alone. Thus 4×10^{-6} M. KCN gave an inhibition of 56%; 42×10^{-3} M. isobutylurethane gave an inhibition of 8%; the combined inhibition amounted to 40%. Finally, in low concentrations of cyanide and high concentrations of narcotic the inhibition of the two was found to be equal to but not less than that of the narcotic alone. Thus 2×10^{-6} M. KCN gave an inhibition of 32%, 63×10^{-3} M. isobutylurethane gave

¹ Fred & Davenport, 1921.

an inhibition of 69% ; the two together gave an inhibition of 69%. These results harmonise with the view that the narcotic tend to displace the cyanide from the cell surfaces.¹

Further observations led Meyerhof to the conclusion that many of the remarkable properties of the nitrifying organism were to be ascribed to their very high permeability by lipid-soluble substances. Thus the lipid-soluble CO_2 is assimilated whilst the lipid-insoluble carbonate is not. Lipid-soluble ammonia is an inhibitor of oxidation, whilst the lipid-insoluble ammonium salts are comparatively inactive. Furthermore, only those amines which are lipid-soluble resembled ammonia in their inhibitive action. Among many inorganic anions borate were found to be the most strongly inhibitive in agreement with the lipid-solubility of boric acid. Finally, the lipid-soluble mercuric chloride was found to be more strongly inhibitory than the lipid-insoluble mercuric nitrate.

The intermediate metabolism of the nitrifiers

The mechanism by which the energy derived from the oxidation of ammonia or nitrite is employed in the reduction of carbon dioxide is obscure. Klein and Svolba² claim to have shown that formaldehyde is an intermediate product in the reduction process, an observation which was held to bring nitrifying organisms into relation with the green plant. It was calculated from the $\frac{\text{N}}{\text{C}}$ ratio of 35 for *Nitrosomonas* that if all the carbon assimilated passed through the stage of formaldehyde, 19.5 mg. of the latter would be formed during the oxidation of 2 g. of ammonium sulphate. By the use of sulphite and of dimedon as fixatives it was demonstrated that formaldehyde was actually formed, about half the amount theoretically possible being actually obtained in the most favourable experiments. This is seen in Table 5.

TABLE 5³

Nitrite culture, c.c.	Age of culture when fixative was added, days	Fixing material	Duration of action, days	Formaldehyde fixed, mg.
100	6	K_2SO_3 , 5%	7	2
100	13	K_2SO_3 , 1%	9	5
100	13	K_2SO_3 , 2%	9	4
100	10	Dimedon, 1%	12	7

The same authors claim to have obtained acetaldehyde as well as formaldehyde by the fixation method. This they regarded as

¹ Meyerhof, 1916 (2).

² Klein & Svolba, 1926.

³ Ibid.

evidence of the existence of a breakdown as well as a synthesis of carbohydrate. The addition of phenylurethane 0.1% and of cyanide 0.02% was found to suppress the formation of formaldehyde (assimilation process) but to leave the acetaldehyde (regarded as representing the respiration or degradation process) untouched. Both formaldehyde and acetaldehyde, added as the dimedon compounds, disappeared when added to the culture fluid.

With regard to the actual process of oxidation, no experimental data exist. Kluver and Donker¹ have suggested that hydroxylamine and hyponitrous acid may be intermediate products, but this view is as yet unsupported by experimental evidence.

THE OXIDATION OF SULPHUR

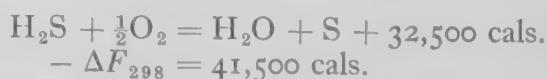
Bacteria which live autotrophically by the oxidation of elementary sulphur and of incompletely oxidised sulphur compounds are very widely distributed and comprise several groups which are sharply distinguished in their metabolic habits. They include, besides, a number of morphological species, but biochemically they fall into five classes. The first is characterised by the intracellular oxidation of hydrogen sulphide to sulphur which is deposited in the form of refractive granules inside the cells; on the supply of hydrogen sulphide falling off the sulphur granules disappear, being in their turn oxidised to sulphate. The second group differs from the first in its ability to oxidise not only hydrogen sulphide but also thiosulphate and tetrathionate; the sulphur is deposited outside the cell. The third group effects the oxidation of sulphur by means of nitrate, elementary nitrogen being liberated. The fourth group oxidises sulphur and thiosulphate direct to sulphate with no deposit of elementary sulphur; it is characterised by high acid tolerance and low optimum pH. The fifth group resembles the fourth in mode of oxidising sulphur but has a high pH optimum and lower acid tolerance; it is facultatively heterotrophic. The sixth group does not fall into the category of autotrophic organisms; thiosulphate is oxidised to tetrathionate, but carbon dioxide is not simultaneously assimilated and the organisms are obligatorily heterotrophic; they form a link between autotrophs and heterotrophs.

Group I. Organisms depositing intracellular sulphur

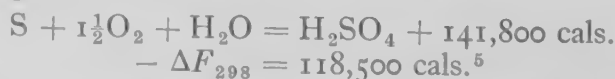
Members of this class were originally discovered in thermal springs containing hydrogen sulphide, and later in stagnant water, both salt and fresh, in which hydrogen sulphide was produced by putrefactive bacteria. The first step in the elucidation of their

¹ Kluver & Donker, 1926.

metabolism was the observation made by Cramer¹—and later confirmed by Cohn²—that the refractive granules contained in *Beggiatoa* could be extracted by carbon bisulphide or by absolute alcohol, and consisted of elementary sulphur. Cohn rightly surmised that the deposition of sulphur in the bacterial cell was the result of the partial oxidation of the hydrogen sulphide. H. and his contemporaries erroneously believed, however, that the hydrogen sulphide was produced by the same organisms as were concerned in its oxidation. The main outlines of the physiology of this group were disclosed by the work of Winogradsky;³ using the type *Beggiatoa* he showed by cultivation in the moist chamber of the microscope that, in the presence of hydrogen sulphide and air, intracellular granules of sulphur were deposited, but that when the supply of hydrogen sulphide fell off the sulphur disappeared and sulphate appeared in the culture fluid; after the disappearance of the intracellular sulphur the organisms died off. He also demonstrated that *Beggiatoa* was not the agent concerned in the production of hydrogen sulphide from sulphate by showing that the disappearance of the sulphur granules took place as readily in culture fluids supplied with sulphate as in its absence. Winogradsky did not prove conclusively that *Beggiatoa* depended solely on the oxidation of hydrogen sulphide for its energy requirements. He showed, however, that the organism developed best when organic material was at a minimum, and that carbon dioxide or carbonate was essential for growth. The absence of complete proof of the autotrophic character of *Beggiatoa* was due to the fact that Winogradsky never succeeded in obtaining the organism in pure culture. This was later achieved by Keil,⁴ who, working with *Beggiatoa* and *Thiothrix*, proved that development took place in the complete absence of organic carbon compounds. It seems, therefore, that the bacteria of this group depend for their energy requirements on the oxidation of hydrogen sulphide:



On the supply of hydrogen sulphide falling off, oxidation of intracellular sulphur occurs:



Group II. Organisms oxidising sulphur compounds with extra-cellular deposition of sulphur

The existence of the second group of sulphur bacteria was

¹ See Müller, 1870.

² Cohn, 1875.

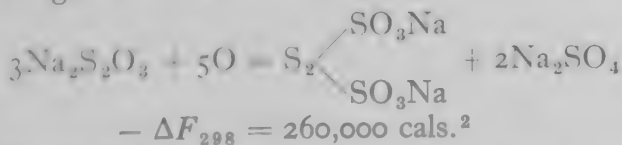
³ Winogradsky, 1887.

⁴ Keil, 1912.

⁵ Baas-Becking & Parks, 1927.

discovered by Nathansohn¹ during an attempt to obtain *Beggiatoa* in pure culture. He found his organism in sea water to which potassium sulphide had been added, and isolated it in pure culture from agar plates made from the same medium; it was subsequently known as *Thiobacillus thioparus*. The culture was characterised by a deposition of sulphur outside the cell, whereas intracellular sulphur was never observed. It developed freely in sea water containing 0.1–1.0% of sodium thiosulphate or in synthetic medium made slightly alkaline with magnesium carbonate. Nathansohn showed that his bacterium developed in the complete absence of organic food material, which did not, however, appear to hinder development. Like the nitrifiers, *Thiobacillus thioparus* was shown to depend on carbon dioxide for its carbon supply, no development taking place, either in the presence or absence of organic compounds, in media from which both carbonates and free carbon dioxide were excluded.

Since the deposition of sulphur by this organism was found to occur outside the cell, Nathansohn experienced a difficulty in explaining how the energy liberated could be used for the intracellular reduction of carbon dioxide on which the organism appeared to depend for its growth. He therefore undertook quantitative studies on the action of the bacillus on sodium thiosulphate which might disclose the occurrence of intermediate stages in the oxidation. A 2% solution of sodium thiosulphate containing an actively growing culture was left until the thiosulphate had nearly disappeared. Three estimations were then made: (1) the sulphate in the solution (A); (2) the unused thiosulphate in terms of sulphate (B). The filtrate from (A) was then completely oxidised with bromine water; any unoxidised compounds of sulphur (including (B)) were thus obtained as sulphate (C). It was found in every case that (C) largely exceeded (B). Nathansohn deduced from this that there must be present some soluble compound of sulphur due to the partial oxidation of the thiosulphate. The figures actually obtained pointed to the conclusion that the oxidation of the thiosulphate proceeded through the stage of tetrathionate:



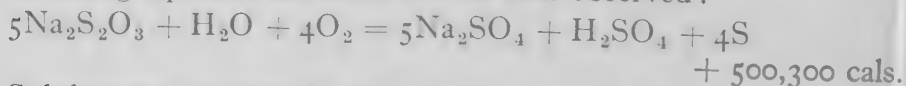
Such a reaction carried to completion would result in the ratio $\frac{A}{C-B} = \frac{1}{2}$; the figures actually obtained were $\frac{33.2}{66.8}$ which is

¹ Nathansohn, 1902.

² Baas-Becking & Parks, 1927.

in close agreement with the supposition that tetrathionate is the intermediate compound formed.

Subsequent work showed that Nathansohn's bacillus was a member of a widely distributed group found in water, mud and soil. Beijerinck¹ obtained from fresh (canal) water, using a synthetic medium, a bacillus which he called *Thiobacillus thioparus*,² able to oxidise calcium and hydrogen sulphide, thio-sulphate and tetrathionate, with the deposition of sulphur outside the cell and the production of sulphate. Nitrogen could be supplied either by ammonium salts or by nitrate, and carbon dioxide (or bicarbonate) was essential as a source of carbon. Identical or closely related organisms are widely distributed. Jacobsen³ isolated them from mud, sewage and sea water, Starkey from agricultural soil, and other observers from waters and soils of widely differing localities.⁴ Starkey⁵ showed that *Thiobacillus thioparus* oxidises thiosulphate to sulphate, but he found no intermediate formation of polythionates; he suggested that their appearance in Nathansohn's experiments may have been due to contaminants of the type of Trautwein's bacillus (see below). During the course of the oxidation the thiosulphate disappearing was accounted for as sulphur and sulphate, the ratio of elementary and sulphate sulphur remaining approximately constant (2/3) throughout the experiment, whilst the medium became progressively acid, reaching in one case pH 4.4. The following equation accounts for the facts observed:

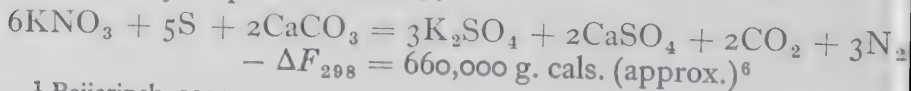


Sulphur was oxidised by this organism to sulphate, but the oxidation was slow in comparison with the oxidation of thiosulphate and hence does not seriously invalidate the above equation.

The strictly autotrophic nature of this organism, which nevertheless can develop in the presence of organic food material, was confirmed by Starkey.

Group III. Organisms oxidising sulphur and sulphur compounds by means of nitrate

Beijerinck isolated an organism (*Thiobacillus denitrificans*) capable of effecting denitrification at the expense of the energy produced by sulphur oxidation:



¹ Beijerinck, 1904.

² The name was first given to Beijerinck's bacillus, though the discovery of the group was due to Nathansohn.

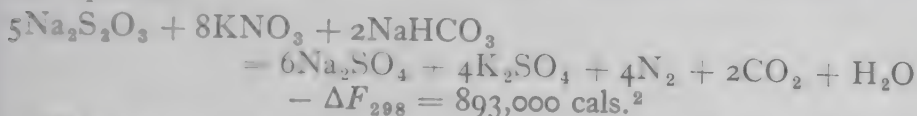
³ Jacobsen, 1912 and 1914.

⁴ Starkey, 1935 (1).

⁵ Ibid. (2).

⁶ Baas-Becking & Parks, 1927.

This bacillus was cultivated on a synthetic medium (No. VI) at 30°. Lieske,¹ using medium No. VII, isolated from pond mud a similar organism which oxidises thiosulphate according to the equation



This organism fixed 1 g. of carbon per 100 g. of sodium thio-sulphate decomposed; hence

790 g. $\text{Na}_2\text{S}_2\text{O}_3$ liberates 893,000 cal.

1 g. $\text{Na}_2\text{S}_2\text{O}_3$ liberates 1130 cal.



(3×10^{-4} atm.) (0.2 atm.)

$-\Delta F = 708,900 \text{ cal.}$

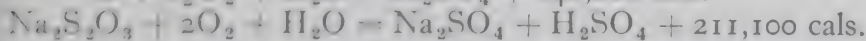
Hence 1 g. carbon requires $\frac{708,900}{72} = 9846$ cal. for fixation,

which is supplied by the oxidation of 100 g. of sodium thiosulphate liberating 1130×100 cal. Hence the free energy efficiency of the organism

$$= \frac{9846 \times 100}{1130 \times 100} \% = 8.7\%$$

Group IV. Very acid-resistant organisms oxidising sulphur and sulphur compounds direct to sulphate

This group was discovered by Waksman and Joffe³ in soils containing free sulphur and rock phosphate. The bacterium, known as *Thiobacillus thio-oxidans*, was isolated by repeated transfer into liquid media (No. VIII) containing powdered sulphur and having an acid reaction; its metabolism was closely studied by its discoverers and also by Starkey.^{4, 5} In common with other sulphur bacteria, *Thiobacillus thio-oxidans* was found to be strictly aerobic, depending solely on sulphur oxidations for its energy requirements. Sulphur or thiosulphate was oxidised directly to sulphate with no deposition of sulphur or polythionates.



The mechanism by which solid particles of sulphur gain access to the cell has been elucidated with this organism,⁶ which contains an oily drop at each end of the cell. The cell moves around in the medium until it strikes a sulphur particle which adheres to the droplet in which it dissolves. Dried cells extracted with acetone

¹ Lieske, 1912.

² Beijerinck, 1920.

³ Waksman & Joffe, 1922.

⁴ Starkey, 1925 (1), (2).

⁵ Waksman & Starkey, 1922.

⁶ Umbreit, Vogel & Vogler, 1942.

followed by ether are found to contain 9-10% of unsaturated oil (iodine number 212), which appears to correspond with these droplets.

The organism is strictly autotrophic, carbon dioxide forming the sole source of carbon, the energy for synthesis being supplied by the sulphur oxidations. The most unusual feature of this bacillus was found in its tolerance for the sulphuric acid which it produces, its optimum pH lying between 3 and 4, whilst the cell survives even a pH of 0.6. Even so, however, the rapid production of sulphuric acid calls for the use of a buffer in the medium, the most convenient being tricalcium phosphate, which reacts with the sulphuric acid to give an acid salt and calcium sulphate:



The influence of organic compounds on the development of this organism calls for special note. Waksman and Joffe found that glycerol, mannitol and glucose slightly favoured the oxidation of sulphur, but that none of these compounds could replace carbon dioxide as a source of carbon. Starkey found that glucose could be tolerated up to 5%, and was indeed actually broken down by the bacteria; though it could not replace sulphur as a source of energy its presence seemed to favour slightly the ratio of carbon fixed to sulphur oxidised. We have here an instance of an organism able to break down glucose but unable to derive from the process either the carbon or the energy required for growth.

Ammonium salts were found to be the best source of nitrogen for this organism. Waksman and Joffe stated that nitrates and amino-acids could also be used, but this was later contradicted by Starkey, who found that nitrates were injurious and that nitrites were toxic in concentrations of 1.25%. Peptone also was found highly injurious.

The energy exchange of *Thiobacillus thio-oxidans* was calculated by Waksman and Starkey.¹ The organism was grown on a medium (No. VIII) containing ammonium sulphate and 1% of powdered sulphur. The disappearance of the sulphur and the production of sulphate were followed gravimetrically, and the carbon of the organism by a wet combustion; the sulphur carbon ratio thus obtained $\frac{(\text{sulphur oxidised})}{(\text{carbon assimilated})}$ was 31.82.

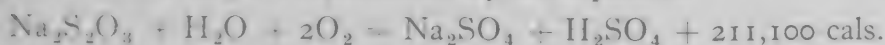
1 g. carbon requires for assimilation	9,846 cal.
32 g. sulphur on oxidation liberates	141,800 cal.
31.82 " " "	140,900 cal.

Hence the energy efficiency = $\frac{9846 \times 100}{140,900} \% = 6.2\%.$

¹ Waksman & Starkey, 1922.

Group V. Organisms oxidising thiosulphate to sulphate, moderately tolerant of acid

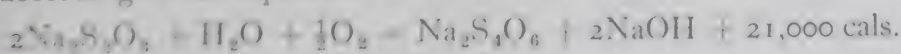
An organism of this type known as *Thb. novellus* was isolated by Starkey from soil. Like *Thb. thio-oxidans* it oxidises thiosulphate to sulphate with no intermediate formation of polythionates or elementary sulphur. It is a true autotrophant, being able to develop on a synthetic medium with thiosulphate as a source of energy and to synthesise significant amounts of cell carbon (1.25 mg. C/100 ml. medium in 43 days). The ratio S oxidised carbon assimilated amounted on an average to 56. The reaction may be represented by the equation



Adopting the same method of calculation as for *Thiobacillus thio-oxidans* the energy efficiency of *Thb. novellus* is 5.3%. *Thb. novellus* differs from *Thb. oxidans* in its pH optimum, which is on the alkaline side (8.0-9.0), and in its much slighter tolerance for acid, pH 5.0-5.5 sufficing to check growth. It is also a facultative autotrophant developing prolifically on organic media; in these conditions power to oxidise thiosulphate is not developed proportionately to growth.¹

Group VI. Intermediate organisms oxidising thiosulphate but living heterotrophically

Members of this group were first described by Trautwein² from sewage effluent. When in pure culture the organism oxidised thiosulphate to polythionates and no deposition of sulphur occurred. Trautwein believed his organism to be autotrophic because, when sown into inorganic medium, thiosulphate failed to disappear in an atmosphere free from CO₂; in these conditions, however, growth and oxidation of thiosulphate took place when carbon compounds were added and galactose and maltose were shown to be utilised. From these observations the organism was regarded as a facultative autotrophant. The question was reopened by Starkey using two of Trautwein's strains and a similar organism isolated by himself. He showed that the oxidation of thiosulphate is attended by increasing alkalinity. All the thiosulphate oxidised was recovered as polythionate, the principal product being tetrathionate; probably the tri- and pentathionate also found to occur as secondary products were due to the increasing alkalinity of the medium. The reaction probably proceeds according to the equation



¹ Starkey, 1934 (1), (2).

² Trautwein, 1921 and 1924.

Although these organisms, when sown into inorganic medium in amounts corresponding to 1.5 mg. C/100 ml., effect the oxidation of the thiosulphate, no increase of bacterial carbon occurs.¹ The addition of organic compounds results in cell multiplication and consequently increased oxidation of thiosulphate. There is thus very strong evidence for regarding these organisms as heterotrophants which happen to be capable of oxidising thiosulphate. This view is strengthened by the fact that three undoubted heterotrophants *Ps. aeruginosa*, *Ps. fluorescens* and *Achromobacter stutzeri* were found to behave towards thiosulphate in all respects exactly like the Trautwein strains. Prolonged cultivation on organic media in the absence of thiosulphate results in no diminution of power to oxidise this substance. From the equation given above it is seen that this type of oxidation gives less energy than the oxidations previously described and therefore is less adapted to an autotrophic mode of life.

The relation of this group to the sulphur autotrophants on the one hand and to the heterotrophants on the other is a matter for interesting conjecture. They may indicate a link in the passage of autotrophic to heterotrophic life or vice versa; they may, on the other hand, represent the fortuitous acquisition by the heterotrophic organisms of an additional oxidising mechanism which happens to be of an inorganic material.

THE OXIDATION OF THIOCYANATE

Happold and Key² isolated an organism (*B. thiocyan oxidans*) from gas-works liquor treated with 20 times its volume of sewage effluent and passed through filter beds. This decomposes ammonium thiocyanate according to the equation



It can be grown on a synthetic medium with ammonium thiocyanate and nitrate as sole source of C and N and also on broth; on the latter medium it does not attack thiocyanate. There is some evidence that an adaptive enzyme is concerned in the oxidation of the thiocyanate.

THE IRON BACTERIA

Among iron bacteria are classified those organisms which, when cultivated in media containing iron salts, accumulate in their outer sheath a deposit of iron consisting mainly of ferric hydroxide; it is to this phenomenon that the yellow-red colour of many streams and natural waters is due. The same natural

¹ Starkey, 1935 (2).

² Happold & Key, 1937.

process frequently leads to much trouble in conduits and pipes through which iron-containing waters flow, the organisms causing the accretion of deposits of iron, incrustations resulting in the blocking of pipes, and also rendering the water unpleasant in appearance and taste. Iron bacteria belong for the most part to the class of thread bacteria (e.g. *Leptothrix*), though other forms such as *Gallionella* are frequently associated with them; the deposit of a hard insoluble material in the sheaths sometimes causes these organisms to assume very peculiar morphological forms, such as the twisted hairpin form of *Gallionella* and the spiral ribbon structure of *Spirophyllum ferrugineum*.¹

The metabolism of Leptothrix ochracea

The first serious study of the physiology of the iron bacteria was due to Winogradsky.² He obtained his culture from chopped hay suspended in a cylinder of spring water to which ferrous hydroxide was added, from which the bicarbonate was formed by passing in carbon dioxide. In this medium *Leptothrix* developed freely, depositing much insoluble or ferruginous material in the sheath and reproducing by "swarming." The phases of growth were watched in the moist chamber of the microscope and in watch glasses. Oxidation of the ferrous salts was shown to occur aerobically, even in the absence of bacteria, to a depth of about 0.5 mm. In the presence of the iron bacteria, this process is extended to a depth of 1-2 mm., indicating that at lower tensions of oxygen the oxidation depends on the agency of the organism. The growth of *Leptothrix* in this medium—which is of indefinite composition but low carbon content—depends on the presence of ferrous carbonate (or bicarbonate); if this is absent or previously oxidised to the ferric condition, no development occurs. The ferrous carbonate is shown to be readily absorbed by the cell and oxidised first to a soluble salt, which is then deposited in the sheath of the cell as ferric hydroxide. At the end of 24 hours the mere passage of carbon dioxide suffices to dissolve the sheath, leaving the cells only faintly coloured; later the deposit assumes a more insoluble—probably more basic—form, and is only with difficulty soluble in dilute hydrochloric acid. *Leptothrix ochracea* forms prolific growth in waters of very low carbon content, and deposits quantities of iron many times the weight of the cell. Winogradsky, from the first, regarded its growth as autotrophic and held that the development depended on the assimilation of carbon dioxide by means of the energy liberated by the oxidation of ferrous to ferric iron. His evidence for this was, however, incomplete; his cultures were doubtless

¹ Ellis, 1919.

² Winogradsky, 1888.

growing in media of very low organic carbon content and, in these circumstances, were dependent on the presence of ferrous iron. The organism was, however, not obtained by him in pure culture, nor was it shown by him to develop in inorganic media from which organic carbon was strictly excluded. As a consequence of this lack of rigid proof, subsequent workers denied the validity of Winogradsky's conclusions, and considerable confusion grew up around the subject.

One of the principal opponents of Winogradsky's views was Molisch, who himself made important contributions to the study of the group.¹ He was the first to obtain *Leptothrix ochracea* in pure culture, and showed that it is not a strict autotrophant, but can grow in peptone, with or without iron. Furthermore, he showed that if in such media manganese replaces iron, the oxide of the former metal is deposited in the sheath of the organism. From these observations he concluded that iron plays no essential part in the metabolism of this organism, and that the deposit of oxides of iron (or manganese) in the sheath is due to adsorptive processes and has no connection with any metabolic function, being, in fact, paralleled by iron accretions in certain *algæ*, moulds, *infusoria* and flagellates where no physiological role is assigned to it. Similar views have been adopted by other workers in this field, notably by Ellis, in an interesting descriptive work on the action of iron bacteria in water tanks and mains.² In spite of the importance of Molisch's observations, it is, nevertheless, clear that if Winogradsky's work failed to supply adequate proof of the autotrophic character of *Leptothrix ochracea*, Molisch's results were equally inadequate for disproving it, the fact that *Leptothrix* can grow heterotrophically on peptone in the absence of iron being no proof that, in its presence, and failing an adequate supply of organic carbon, the same organism may not be capable of autotrophic development.

Lieske³ next reopened the subject, and has done much to clear away the prevalent confusion respecting the metabolism of these organisms. He obtained *Leptothrix* in pure culture (without the use of the peptone employed by Molisch) on a solid medium containing agar and manganese acetate (No. X). The organism thus isolated developed only very feebly or not at all on ordinary laboratory culture media. In peptone 0.1% very sparse growth was obtained, which was improved in peptone concentrations of 0.5 to 2%. In the latter medium improved growth was obtained by the addition of manganese carbonate. In media approaching in character the marshy waters which are the natural habitat of the organism, such as weak aqueous extracts of leaves or peat, the

¹ Molisch, 1910

² Ellis, 1919.

³ Lieske, 1919.

addition of manganese carbonate markedly increased the growth, thus indicating that in the absence of an adequate supply of organic carbon the cell depends on the oxidation of the metallic salts.

The organism was also cultivated in strictly autotrophic conditions (medium No. X). In these circumstances the manganese carbonate (which Lieske used in place of iron) was found to be essential for development, as was also the presence of atmospheric carbon dioxide. Whether the importance of the latter is to be attributed to its effect on the reaction of the media, and hence on the solubility of the carbonate, or whether it has a direct effect on the assimilation process, was not determined.

The metabolism of Spirophyllum ferrugineum

As in the case of the sulphur bacteria, the metabolism of the iron bacteria differs from species to species, and it is not possible to assume that facts relating to one form are true of another. The metabolism of *Spirophyllum ferrugineum*, an organism usually found in the form of a ribbon-like spiral,¹ has received a detailed study by Lieske² in a paper which has contributed much to exact knowledge on the subject of iron bacteria. The organism was obtained from a ferruginous stream and was first cultivated in its natural water, to which, besides old leaves, iron wire was added, and after several transfers it was finally obtained in pure culture and cultivated in synthetic medium (No. XII), and its strictly autotrophic character fully established. The growth of the organism was found to depend on the presence of iron, which (as distinct from the case of *Leptothrix ochracea*) could not be replaced by manganese nor by any other metal tried (lead, tin, bismuth, cadmium, zinc, nickel, tungsten, chromium, magnesium, or copper). The iron appeared to be absorbed in the form of ferrous bicarbonate; this could be supplied either by iron wire, which in an atmosphere containing 1% carbon dioxide yields the bicarbonate, or by adding small quantities of reduced iron and passing through carbon dioxide. Ferrous bicarbonate (0.01%) could also be added direct; in the latter case, as soon as the ferrous bicarbonate was oxidised, growth ceased until the supply was renewed. Other iron salts (ferric chloride, ferrous sulphate) did not replace the ferrous bicarbonate. Subservient to this necessity, carbon dioxide is an essential requirement of the organism, no growth being obtained in an atmosphere free from carbon dioxide and increased growth if the atmospheric concentration is raised to 1%.

This organism, unlike *Leptothrix ochracea*, is very strictly auto-

¹ Ellis, 1919.

² Lieske, 1911.

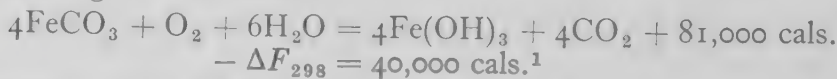
trophic and will not develop in the presence of organic compounds, 0.25% peptone or cane sugar or 0.35% asparagine producing total inhibition, some depression of growth occurring even at 0.01%.

The optimum temperature of the organism was found to be remarkably low, as the following figures show :

Temperature	Growth of <i>Spirophyllum</i>
0.0 to 0.5	Good
6°	Excellent (best)
15°	Good
22°	Slight
27°	None
32°	None

In accordance with these results it was found almost impossible to obtain cultures of this organism from its native streams during the summer months, its place being then taken by copious growths of *Leptothrix ochracea*.

The energy required for the growth of the organism is supplied according to the reaction



Assuming that the free energy efficiency of the organism is 5% (about the average for the autotrophic bacteria), the synthesis of 0.5 g. of carbon would require the oxidation of 224 g. of ferrous iron.

It has been pointed out by Starkey² that starting from the above equation 10 K. cal. are liberated by the oxidation of 55.8 g. iron in the form of ferrous carbonate. Assuming that the iron bacteria have an efficiency of 8%, i.e. 8% of the free energy liberated is used for the synthesis of cell material from CO_2 , then the oxidation of 55.8 g. ferrous iron would result in the assimilation of $\frac{0.8}{115} \times 12 = 0.0835$ g. carbon, representing approximately

0.21 g. organic material. Thus from the oxidation of 55.8 g. ferrous iron would be formed 106.8 g. ferric hydrate and 0.21 g. cell material, a ratio of 500 to 1. It is therefore apparent that wherever iron bacteria are growing autotrophically very large deposits of ferric hydrate may be expected.

In cases where heterotrophic organisms are metabolising iron in demonstrable quantities (as shown by the Prussian blue reaction) this large ratio does not occur. Pringsheim has described iron flagellates growing heterotrophically and incapable of growing autotrophically which, in certain circumstances, metabolise considerable quantities of iron and may deposit iron and/or manganese in particular situations—as, for example, the stalks of *Anthophysa*.³

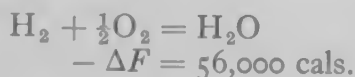
¹ Baas-Becking & Parks, 1927.

³ Pringsheim, 1946.

² Starkey, 1945 (1), (2).

ORGANISMS OXIDISING FREE HYDROGEN

The gap between autotrophic and heterotrophic bacteria is not so great as at first appears, since several species are known connecting the two types. Important among these are certain soil organisms ("Knallgasbakterien") able to use molecular hydrogen as a source of energy, whilst deriving their carbon solely from carbon dioxide. The first of such organisms to be described was *B. pantotrophus*, discovered by Kaserer.¹ Flasks containing an inorganic medium (No. XIII) were inoculated with soil and filled with a mixture of air, carbon dioxide and hydrogen. From the growth so obtained, Kaserer isolated an organism (*B. pantotrophus*) which could either grow autotrophically by means of the energy obtained in the reaction

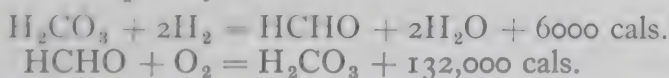


or heterotrophically on ordinary media.

Other organisms having the same type of metabolism, but very various forms (large and small bacilli, both sporing and non-sporing and cocci), were subsequently described, and all soils investigated, with the exception of that from sand dunes, yielded this physiological type.² One of the sulphate reducers has been found able to live anaerobically by reducing sulphate to sulphide by molecular hydrogen. This is an anaerobic autotrophant.³

Intermediate metabolism of hydrogen bacteria

Kaserer showed that his organism was able to tolerate and use formaldehyde in a concentration of 1 in 20,000. This observation led him to the conclusion that the hydrogen was not oxidised direct, but that carbon dioxide was first reduced to formaldehyde, which was subsequently oxidised:



Ruhland,⁴ working with *B. pycnoticus*, has added considerably to our knowledge of the metabolism of the hydrogen oxidisers. Using medium No. XIV, the optimum pH for the oxidation lay between 6.8 and 8.1, oxidation ceasing outside the limits pH 5.2 and 9.2. The rise in rate from pH 5.2 to 6.8 was found to be parallel with a rise in the ratio $\frac{\text{HCO}_3'}{\text{CO}_2}$ from 0 to 6.0, whilst the fall on the alkaline side corresponded to the reaction at which iron

¹ Kaserer, 1906.

² Nishikawa & Lebedeff, 1906; Grohmann, 1924; Ruhland, 1924.

³ Butlin, 1947.

⁴ Ruhland, 1924.

salts were precipitated. The relative pressures of hydrogen and oxygen had little effect on the rate of oxidation. By a series of gas analyses during the course of the development of the organism, it was found that the ratio hydrogen/oxygen used was not 2, as would be the case if all the hydrogen used was oxidised, but considerably in excess of this figure. This is explicable on the supposition that part of the hydrogen is being burnt direct whilst part is being synthesised into cell material. Regarding for simplicity the composition of the latter as being represented by carbohydrate, the following equations give the volume of gas reacting:



If the carbohydrate (representing cell material) is subsequently burnt



One volume of carbon dioxide formed represents two volumes of hydrogen passing into synthesis. Ruhland oxidised the cell material formed in his experiments by means of a wet combustion method, and proved this relationship to hold within the limits of his experimental error (see Table 6, columns VII and VIII).

TABLE 6

I	II	III	IV	V	VI	VII	VIII
Duration of exp., days	Vol. of medium, c.c.	Vol. of H ₂ used, c.c. A	Vol. of O ₂ used, c.c. B	$\frac{\text{H}_2}{\text{O}_2}$	Excess of H ₂ A - 2B C	$\frac{\text{C}}{2}$	Vol. of CO ₂ obtained by combustion of cell material
6	50	137.82	52.75	2.63	32.32	16.1	16.9
11	50	111.48	44.72	2.50	22.04	11.0	10.4
14	100	89.48	39.06	2.29	11.02	5.5	6.2

The same set of figures shows that the smaller the relative amount of growth the more closely does the hydrogen/oxygen ratio approach 2, which is in accordance with the view that the excess of hydrogen over oxygen is due to the reducing function of the latter in the assimilation process.

In optimal conditions of pH, temperature, etc., the $\frac{\text{H}_2}{\text{O}_2}$ quotient and hence the $\frac{\text{CO}_2 \text{ assimilated}}{\text{H}_2 \text{ burnt}}$ remained remarkably constant, the former amounting to 2.78 to 2.79, the latter to 0.137 to 0.140, from which it follows that the oxidation of 7 to 8 ml. of hydrogen results in the fixation of 1 ml. of carbon dioxide. Ruhland sought

to determine whether the dual processes of carbon assimilation and hydrogen oxidation could be dissociated. In order to do this, he carried out a series of ten-day experiments in which growth was prevented by employing washed cell suspensions in a medium from which carbon dioxide and carbonate were excluded. The hydrogen oxygen quotient, in these circumstances, was 1.80, which strongly suggests that the hydrogen oxidation was being carried out independently of carbon assimilation. The failure of the quotient to attain the theoretical value of 2 might be attributable to an endogenous oxygen uptake due to the oxidation of cell substance.

The energy efficiency of the organism calculated from Ruhland's data

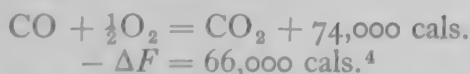
$$= \frac{112.3 \times 100}{69.0 \times 8} = 20.5\%$$

The free energy efficiency from the same data¹

$$= \frac{118,100 \times 100}{8 \times 56,000} = 26.4\%$$

THE OXIDATION OF CARBON MONOXIDE

Comparable to the organisms oxidising hydrogen are those oxidising carbon monoxide. The discovery of these was due to Beijerinck and van Delden,² who isolated from soil an organism which appeared to develop, though slowly, on media from which carbon compounds other than carbon dioxide were excluded. Nitrate, nitrite or ammonia could serve as source of nitrogen, though nitrate had the practical advantage of excluding the nitrifiers. No development took place on ordinary media, the organism being to all appearance strictly autotrophic. Beijerinck and van Delden did not succeed in showing the source from which the cell derived its energy, though they observed that growth occurred more readily in the atmosphere of the laboratory than in the comparatively pure air of a greenhouse. The organism was called by its discoverers *B. oligocarophilus*. Kaserer³ isolated from the soil a bacterium which he identified with *B. oligocarophilus*, and succeeded in showing that it developed by the oxidation of carbon monoxide according to the equation



Lantzsch⁵ studied this organism afresh, and disclosed an inter-

¹ Baas-Becking & Parks, 1927.

² Beijerinck & van Delden, 1903.

³ Kaserer, 1906.

⁴ Baas-Becking & Parks, 1927.

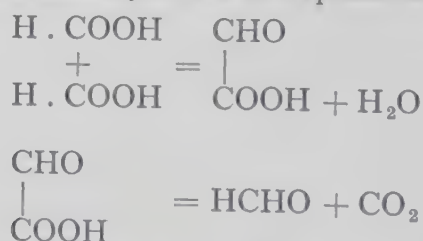
⁵ Lantzsch, 1922.

esting case of polymorphism associated with physiological function. In strictly autotrophic conditions (medium No. XV) with carbon monoxide or formic acid (the latter supplied by exposing the organism to an atmosphere kept saturated with the vapour), the growth obtained was characteristic of the *actinomyces* form, i.e. a network of threads. If the carbon was supplied as formaldehyde, acetone or butyric acid, or if the organism was cultivated on the usual laboratory media, the *actinomyces* form gave way to a coccid form, the original character being regained on resubcultivating into formic acid or carbon monoxide media. Hydrogen was not oxidised in any circumstances.

By the use of crude soil cultures, Wehmer¹ has succeeded in freeing coal gas from carbon monoxide. Anaerobically carbon monoxide was oxidised and sulphate reduced, though, owing to the cultures being mixed, it is uncertain whether the two processes were linked or whether some unknown oxidising agent was responsible for the oxidation of the carbon monoxide.

THE OXIDATION OF FORMIC ACID AND FORMALDEHYDE

An organism, *B. methylicus*, originally obtained from the air by Loew,² is able to develop in inorganic media on formaldehyde sulphite and on 0.5% sodium formate, also on methyl alcohol; it also lives heterotrophically on ordinary media. Nothing definite is known of the metabolism of this organism which seems closely allied to that of *B. oligocarbophilus*, though the two organisms are morphologically unlike. Loew's suggestion that the formic acid is metabolised via formaldehyde has no experimental foundation:



Possibly the formic acid is metabolised via carbon monoxide, as has been suggested for *B. oligocarbophilus*.

THE OXIDATION OF METHANE

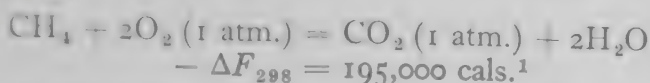
The discovery of an organism (*B. methanicus*) oxidising methane was due to Sohngen.³ This organism undoubtedly exists in natural conditions on the methane produced in anaerobic fermentation in

¹ Wehmer, 1926.

² Loew, 1892.

³ Sohngen, 1906.

the lower layers of the soil. It was cultivated on an inorganic medium in an atmosphere consisting of one-third methane and two-thirds air :



The free energy efficiency of this organism was calculated from Sohngen's data by Baas-Becking and Parks,² and found to be 20.7%. As, however, the figure for carbon assimilated was derived indirectly from the difference between the observed and calculated carbon dioxide obtained from the oxidation of the methane instead of from the combustion of the organism, great accuracy cannot be postulated for this figure.

GENERAL CONSIDERATIONS

From the metabolic point of view, the autotrophic bacteria form a class having characteristics as clearly defined and as distinct as the green plants on the one side and animals (with which may be grouped the heterotrophic bacteria) on the other. Like the plant world and the photosynthetic bacteria the autotrophants are independent of other living organisms, the plants depending on the radiation energy from the solar spectrum, the autotrophants on the energy obtained by the oxidation of inorganic compounds, to enable them to utilise carbon dioxide as a source of carbon for growth. The autotrophants then have more in common with the green plant and with the photosynthetic bacteria than with heterotrophic bacteria and animals, though, as very little is known of the details of their metabolism, we are unable to draw a close parallel.

Regarded from the standpoint of comparative biochemistry, autotrophic bacteria may be regarded as having exploited the possibility of various chemical reactions to supply their need for energy without having hit on anything so profitable as the methods adopted by the green plant on the one hand, or the heterotrophic micro-organisms and the animal world on the other. These two main types of metabolism have, in the course of evolution, turned out to be capable of the enormous development exhibited by the varied forms of plant and animal life, whilst the reactions employed by the autotrophic bacteria appear, in the conditions governing terrestrial life, to be incapable of great expansion or development. It may be noted in passing that the energy efficiency of autotrophic bacteria is low, as may be seen from the accompanying table due to Baas-Becking and Parks (Table 7).³

¹ Baas-Becking & Parks, 1927.

² Ibid.

³ Ibid.

A notable advance in the elucidation of the mechanism involved in this energy transfer has recently been achieved; briefly the following steps have been proved:

By the use of washed suspensions and the manometric technique S oxidation has been separated from CO_2 reduction and the two processes studied separately. Aerobic oxidation of S in the absence of CO_2 has been shown to be accompanied by disappearance of

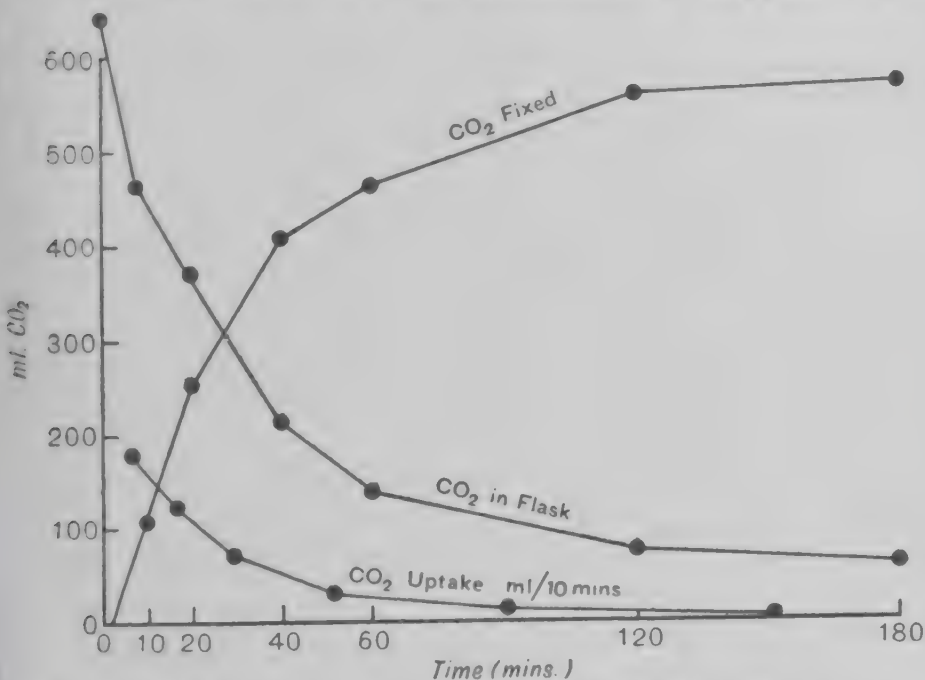


FIG. 5.— CO_2 fixation by resting cells of *Thiobacillus thio-oxidans* in the absence of sulphur¹

inorganic P. At the end of this process the cells are shown to be capable of the anaerobic fixation of CO_2 , a process accompanied by the return of inorganic P to the medium; these complementary processes are shown in Figs. 5 and 6. These observations are of fundamental importance in that S oxidation and CO_2 absorption occur in the absence of cell multiplication and each can be separated from the other. It is shown, however, that although CO_2 absorption can be separated in time from S oxidation yet it is dependent on it. Thus after S oxidation has come to a standstill the cells are able to fix approximately 40 $\mu\text{l. CO}_2/100 \mu\text{g. bacterial N.}$; after this a further period of S oxidation in air restores the cells to a condition in which they are again able to fix CO_2 . Fig. 6 and Tables 8 and 9 illustrate this.^{2, 3}

¹ Vogler, K. G., *J. Gen. Physiol.*, 1942, 26, 109.

² Ibid., 1942.

³ Vogler & Umbreit, 1942.

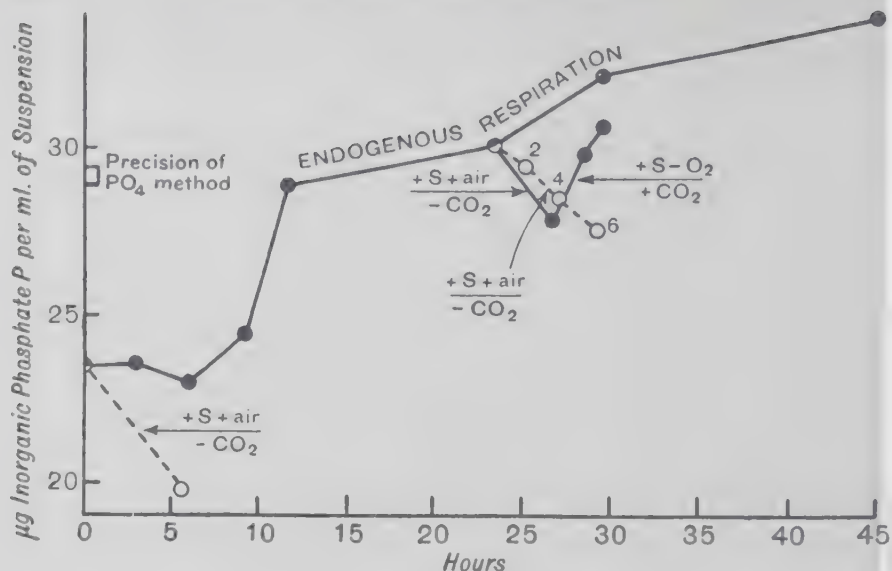


FIG. 6.—Phosphate changes during respiration + CO_2 fixation by *Thiobacillus thio-oxidans*¹

TABLE 8²

INFLUENCE OF THE PREVIOUS OXIDATION OF S ON THE SUBSEQUENT FIXATION OF CO_2

200 µg. bact. N per flask	CO_2 upt. µl.	O_2 upt. µl.	CO_2 upt. µl.
	First period in H_2 2 hours	Period in O_2 2 hours	Second period in H_2 2 hours
1 sulphur absent . .	75	4	0
2 sulphur absent . .	71	4	1
3 + sulphur	70	600	61
4 + sulphur	74	600	56

TABLE 9³

INFLUENCE OF S OXIDATION AND CO_2 FIXATION ON PHOSPHORUS TRANSFER FROM CELLS \rightleftharpoons MEDIUM

Hours :	2	4	6
Bacteria (168 µg. cell N) O_2 upt. µl.	220.0	640.0	1070.0
P transfer from medium to cells µg. P	0.5	2.0	2.5
Gas phase changed from O_2 to CO_2			
CO_2 upt. µl.	32.0	72.0	80.0
P transfer from cells to medium µg. P	1.0	2.2	2.5

¹ Vogler & Umbreit, *J. Gen. Physiol.*, 1942, 26, 158.

² Vogler, 1942.

³ Vogler & Umbreit, 1942.

From the data in Tables 8 and 9 it is found that the CO_2 absorbed in the second period is proportional to the P taken up in the first period, whilst during the CO_2 uptake the P released is proportional to the CO_2 fixed. 70-80 mols. O_2 taken up in the oxidation of S causes the uptake of 1 mol. phosphate, whilst the release of 1 mol. phosphate accompanies the fixation of 40-50 mols. CO_2 . With special precautions to ensure the optimal oxidation of S the following ratios were established :

$\text{O}_2/\text{PO}_4 = 72$; $\text{CO}_2/\text{PO}_4 = 47$; hence $\text{O}_2/\text{CO}_2 = 1.58-1.53$

Assuming that CO_2 goes direct to carbohydrate the expected ratio is 1.49.

Each mol. O_2 used in S oxidation releases 79,000 cals. free energy. Hence $79,000 \times 72$ cals. = 5,688,000 cals. are required for the intracellular uptake of 1 mol. of phosphate.

47 mols. of CO_2 synthesised per mol. phosphate to carbohydrate during the back reaction (liberation of inorganic phosphate) requires the absorption of 5,283,000 cals. free energy, which gives satisfactory agreement with the calories available from the oxidation process. 1 mol. $\text{CO}_2 \rightarrow$ carbohydrate requires 112,400 cals. free energy.

CO_2 as oxidising agent

The CO_2 uptake during S oxidation is a continuous process necessary for the supply of C to the cell. In this case the CO_2 uptake must be accounted for either in terms of an increased level of cell oxidation or by the excretion of oxidised products. If the overall level of oxidation in the cell be about that of carbohydrate



and the CO_2 is converted into cell material, then for 1 mol. CO_2 taken up 1 mol. O_2 will become available (O_2 is never liberated).

TABLE 10¹
 CO_2 AS OXIDISING AGENT IN S OXIDATION

	Q_{O_2}	Q_{CO_2}	$\text{Q}_{\text{CO}_2 + \text{O}_2}$	Time hours
1	1580	0	1580	2
2	1600	0	1600	2
3	1080	710	1730	2
4	1460	283	1773	$\frac{1}{2}$
5	1270	320	1590	2
6	1140	540	1680	2
7	1220	420	1640	2
8	1100	570	1670	2

¹ Vogler, 1942.

If then S oxidation is measured in the presence of CO_2 , and if 1 mol. O_2 is spared for each mol. CO_2 taken up, the Q_0 should decrease in the presence of CO_2 whilst the total volume $\text{O}_2 + \text{CO}_2$ taken up should be constant (see Table 10).

The oxidation and fixation processes are inhibited by different inhibitors (see Table 11).

TABLE 11¹

Inhibitor	% Inhibition	
	S oxidation	CO_2 fixation
CN		
Azide $M/100$. . .	100	0
Arsenite $M/100$. . .	90	0
Iodoacetate $M/10,000$.	10	100
Pyruvate $M/150$. . .	100	100

It has, however, been calculated that the change from inorganic P to A.T.P. requires 10,000 cal., whilst actually about 500 times that amount is supplied by the oxidation of S. It is suggested that there may be a series of P compounds within the cell whose energy value may rise and fall during S oxidation and CO_2 fixation respectively, only the initial and final steps resulting in the appearance or disappearance of inorganic P. Evidence for the presence within the cell of A.T.P., hexose diphosphate, glucose 1-phosphate, glucose 6-phosphate and fructose 6-phosphate has been found.²

¹ Vogler, 1942.² Le Page & Umbreit, 1943.³ Le Page, 1942.

CHAPTER X

BACTERIAL PHOTOSYNTHESIS

THE existence of photosynthetic bacteria has been suspected since 1883 when Engelmann described a pure culture of pigmented organisms with a well-defined absorption spectrum. He concluded that the pigments played an important part in the life of the cell from the fact that irradiation conditioned motility. He furthermore showed that if the spectrum (solar or gas-light) were projected on to the microscopic stage, the organisms collected in the regions of their own absorption bands (Fig. 1). From this

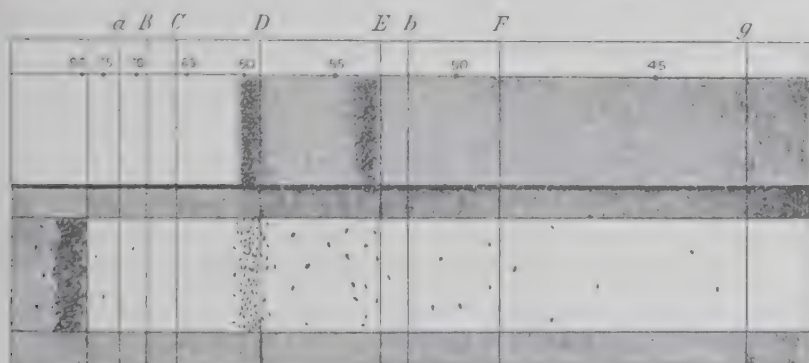


FIG. 1¹

discovery till 1931 the physiology of the purple bacteria fell into great confusion. This is to be attributed to the fact that their development was found to depend on two factors, light and the presence of hydrogen sulphide. Both these observations are fundamental to the understanding of the subject but both played the part of red herrings. The effect of illumination led workers to strive to bring the observed facts into conformity with those known to hold for the green plant. This of course involved the search for free oxygen when the organism was illuminated. Actually Engelmann believed he had demonstrated this by a biological method,² but the error of this observation was shown by Molisch³ and also by recent workers.^{4, 5} The necessity for hydrogen sulphide deflected the course of investigation by suggesting a chemosynthetic type of metabolism in which the hydrogen sulphide pro-

¹ Engelmann, *Pflügers Arch.*, 30, Taf. 1, 1883.

² Ibid.

³ Molisch, 1907.

⁴ van Niel & Muller, 1931.

⁵ Muller, 1933.

vided the energy through oxidation, as Winogradsky had already shown was possible (see p. 256). Such an explanation, however, left the action of light unaccounted for and was contradicted by the fact that the organisms developed anaerobically. The issue was further involved by the observation that some coloured bacteria develop in the dark in the presence of organic material. The task of harmonising these facts and of disclosing the true character of the photosynthetic bacteria was reserved for van Niel, to whose masterly researches and penetrating insight we owe our present knowledge on this subject.

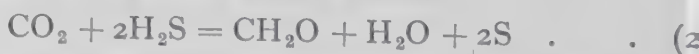
The coloured bacteria were originally found in shallow seas or lagoons exposed to sunshine in localities where a supply of hydrogen sulphide was available. Van Niel¹ showed that they are of wide distribution in the soil and also in fresh and salt water muds and may be cultivated from these sources if the right conditions are complied with. These are :

1. Exposure to light, either sunlight or from an arc lamp.
2. A pure synthetic medium containing the following : NH_4Cl , 0.1% ; K_2HPO_4 , 0.5% ; MgCl_2 , 0.02% ; NaHCO_3 , 0.5% ; $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, 0.1%.
3. pH about 8.5.
4. Strictly anaerobic conditions.

After a preliminary enrichment on a suitable medium, 15 strains of purple and 4 of green sulphur bacteria were obtained in pure culture. These were separated into groups according to the value of pH and concentration of sulphide at which they develop best. By estimations of the CO_2 and H_2S disappearing and the sulphate formed it is found that these are in a stoichiometric relation. In the case of the purple sulphur bacteria this is in accordance with the equation



In the case of the green bacteria, sulphide is oxidised only as far as sulphur, which is deposited outside the cell :



The extent to which van Niel's experiments supported these conclusions is seen in Table 1. With strain 4 the H_2SO_4 produced is equivalent to 264 mols., which, in accordance with the equation requires 528 mols. of CO_2 whereas 505 were actually used. After the sulphide had disappeared no further diminution in CO_2 occurred, showing the interdependence of the two processes. The case of strain 9 gives some information on the course of the process. After 27 days the sulphur had not been completely

¹ van Niel, 1931 and 1935.

oxidised and microscopic examination showed that the cells still contained sulphur globules; correspondingly the CO_2 disappearing was below the amount calculated for equation (1). By 42 days the intracellular sulphur had disappeared and the sulphate formation then corresponded with the amounts of sulphide and CO_2 used.

TABLE 1¹

No. of cult.	Culture		H_2S oxi- dised, mg.	H_2SO_4 produced		CO_2 disappeared	
	Description	Duration in days		Calculated, mg.	Found, mg.	Calculated, mg.	Found, mg.
4	Stores sulphur inside the cell	29	9.14	26.32	25.90	23.63	22.8
9	Stores sulphur inside the cell	{ 27	8.8	25.5	24.7	22.9	20.7
		{ 42	18.7	53.9	51.4	48.4	46.8
1	Does not store sulphur.	24	24.8	71.5	70.9	64.2	63.8
7	Does not store sulphur.	{ 32	11.39	32.83	34.2	29.48	28.5
		{ 36	13.43	38.71	13.72	34.76	17.5

In culture 7 the sulphate formed was also low as compared with the sulphide used. Here this discrepancy was accounted for by an extracellular deposit of sulphur according to the equation (2). Thus out of 395 mols. of H_2S , 255 have been oxidised only to S , corresponding to the reduction of 128 mols. of CO_2 , so that the total maximum reduction of CO_2 would be

$$128 + (2 \times 140) = 408 \text{ mols.}$$

of which 398 were verified experimentally.

The application of manometry to these problems has proved of great advantage. Roelofsen,² using washed suspensions of purple sulphur bacteria, demonstrated an evolution of CO_2 in the dark due to autofermentation and an immediate absorption on irradiation in the presence of sulphide. There appears also an absorption of CO_2 in the absence of sulphide which quickly comes to a standstill; this is doubtless due to some endogenous hydrogen donor whose nature is undetermined (Fig. 2).

A variation on the photosynthetic theme is supplied by the purple bacteria (*Athiorhodaceae*) which are distinguished from the purple and green sulphur bacteria (or *Thiorhodaceae*) by their ability to reduce CO_2 photosynthetically by means of organic compounds,³ and so to develop anaerobically on simple inorganic media with simple organic acids, and CO_2 (or bicarbonate) in the presence of light. It is necessary to distinguish this mode of

¹ van Niel, *Arch. Mikrobiol.*, 1931, 3, 88.

² Roelofsen, 1935.

³ Muller, 1933.

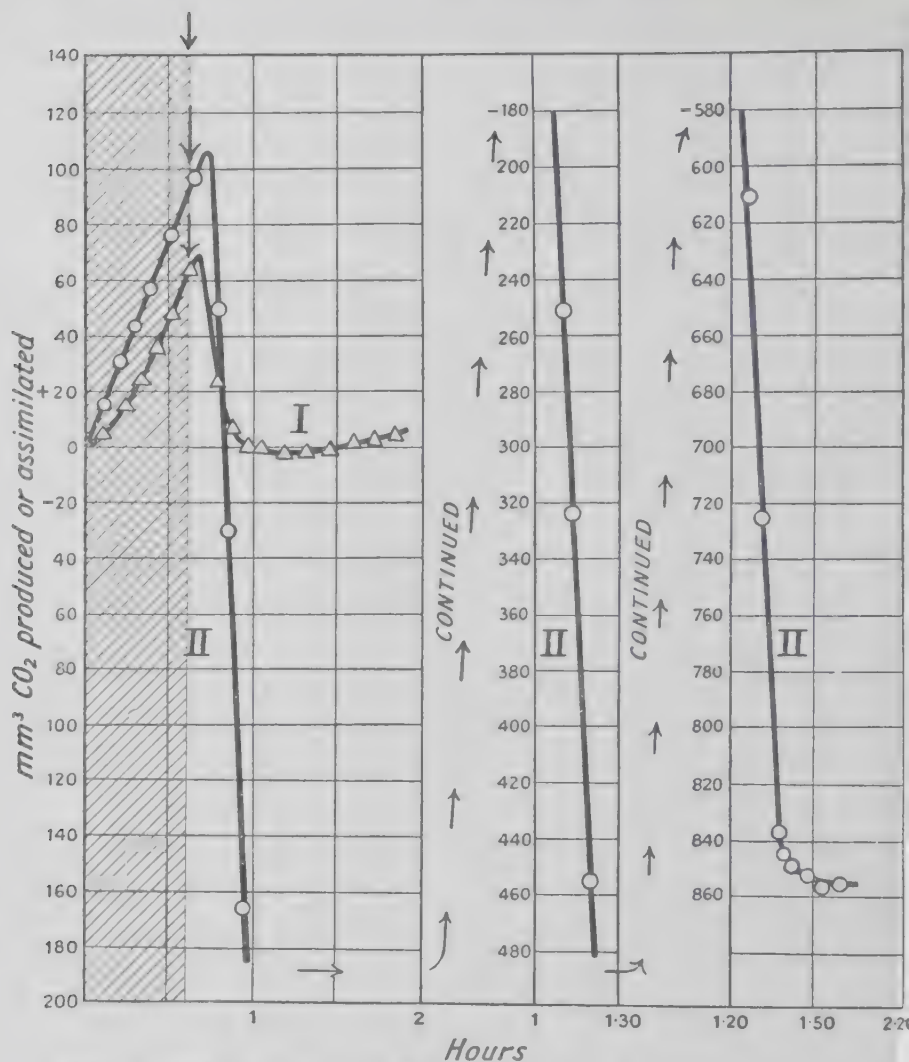


FIG. 2.—CO₂ absorption by *Thiorhodaceae* in dark (shaded) and light, I without and II with H₂S¹

life carefully from the aerobic growth of heterotrophants and from anaerobic growth on carbon compounds. In the first place, this development occurs only in light and is strictly anaerobic; moreover, the organic substrate is broken down with relatively little evolution of carbon dioxide, that is, the substrate passes into the cell material almost quantitatively (Table 2). The true photosynthetic nature of the process is best seen when the substrate consists of a fatty acid such as butyrate which is more highly reduced than carbohydrate and protein and also than the bacterial cell as a whole. Here development does not occur unless

¹ Roelofsen, *Thesis*, p. 78 (1934).

TABLE 2¹

Medium	Substrate-carbon used	CO ₂ -carbon formed	Carbon in bacteria	Total carbon recovered, %
Lactate	3.29	0.41	2.95	102
	1.04	0.12	0.82	90
	3.70	0.25	3.15	92
	4.48	1.25	2.72	89
Malate	5.41	1.77	2.94	87
	2.92	0.86	1.93	96

an extraneous source of carbon dioxide is added. If the various substrates are arranged in order of their oxidation values (or reducibility) the CO₂ produced per millimol of substrate is in the reverse order; this is seen in Table 3.

TABLE 3

Substrate	m. mols. CO ₂	
	m. mols. substrate	
Malate . . .	+ 1.22	
Succinate . . .	+ 0.7	
Acetate . . .	+ 0.17	
Lactate . . .	+ 0.29	
Butyrate . . .	- 0.74	

The same point can be better demonstrated by the use of manometric technique as was done by Gaffron² for the purple bacterium *Rhodovibrio*. Washed suspensions of this organism in the presence of potassium butyrate in an atmosphere of 5% CO₂ in N₂ showed an absorption of CO₂ (in light) proportional to the amount of butyrate present. See Fig. 3. Now in the case of bacteria the photosynthetic process leads to the formation of cell material and, speaking roughly, the degree of oxidation of cell material is about that of protein or carbohydrate, but less than that of fatty acids above acetic. Hence photosynthesis by means of these compounds involves oxidation and this can only occur by absorption of CO₂. Now the longer the carbon chain of the saturated fatty acids the more reduced it becomes and the greater the amount of oxidation, i.e. CO₂ absorption, required to bring it to the oxidation level of cell material. Gaffron showed manometrically that the greater the number of CH₂ groups per mol. of fatty acid the greater the amount of CO₂ absorbed per mol. (see Table 4). More direct evidence of the role of organic compounds in the photo-reduction of CO₂ is shown by those members of the

¹ Muller, *Arch. Mikrobiol.*, 1933, 4, 131.² Gaffron, 1933.

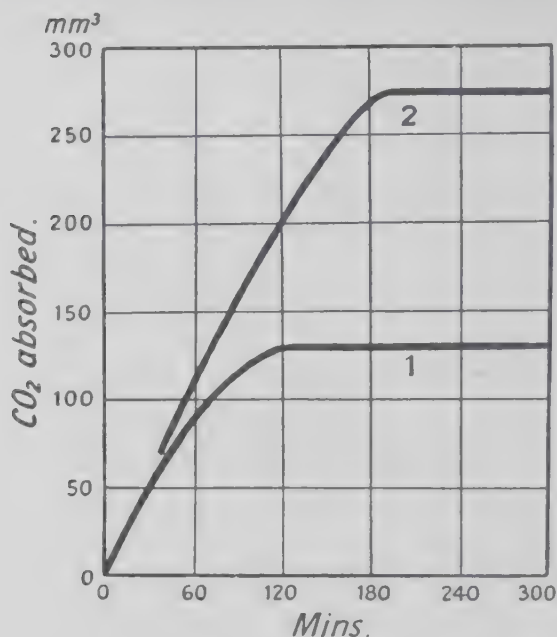


FIG. 3.—CO₂ absorbed in presence of (1) 1 ml., (2) 2 ml. M/100 butyrate¹

TABLE 4²

RELATION BETWEEN YIELD OF CELL MATERIAL AND FATTY ACID USED AS SUBSTRATE

Fatty acid	M. wt. of Na- salt	mg. carbon per m mol.	CO ₂ uptake per m mol.		mg. carbon in cell material from 1 g. substrate
			m mol.	mg. carbon	
Formic acid .	68	12	-0.6	-7.2	75
Acetic acid .	82	24	-0.2	-2.4	230
Propionic acid	96	36	0.3	3.6	350
Butyric acid .	110	48	0.65	7.8	435
Valeric acid .	124	60	1.0	12.0	500
Caproic acid .	138	72	1.4	16.8	550
Heptylic acid .	152	84	1.7	20.4	600
Caprylic acid .	166	96	2.0	24.0	635
Pelargonic acid	180	108	2.4	28.8	665

Athiorhodaceæ able to oxidise isopropanol to acetone. This compound undergoes no further change and can be recovered quantitatively. It has been shown manometrically that 6.0 m. mols. of isopropanol oxidised result in the reduction of 90 µl. CO₂. If the CO₂ were reduced to the carbohydrate level 90 µl. would disappear, hence the results suggest that the reduction products are on an average more reduced than carbohydrate.³

¹ Gaffron, H., *Biochem. Z.*, **260**, 4.

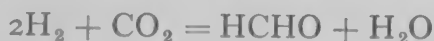
² van Niel, 1944, *Bact. Rev.*, **8**, 37.

³ Foster, 1940.

Members both of the *Thiorhodaceæ*¹ and the *Athiorhodaceæ*^{2, 3} can use organic hydrogen donators and molecular hydrogen; in fact their capacities seem to vary according to the dehydrogenases present just as does the reduction of other hydrogen acceptors in the case of heterotrophic bacteria.

The use of hydrogen as the reducing agent in place of organic compounds in the case of the *Athiorhodaceæ* provides the final and convincing evidence that these organisms build their cell material anaerobically by the reduction of carbon dioxide in light and do not depend on any fermentative breakdown.

An interesting link between the bacterial and green plant method of photosynthesis is provided by certain green *algæ*, notably *Scenedesmus*.⁴ These are amongst the rare examples of organisms other than bacteria able to metabolise molecular hydrogen. Normally they photosynthesise like green plants with the elimination of oxygen. If kept anaerobically in hydrogen and carbon dioxide for one hour or longer at low intensity of illumination their metabolism changes and hydrogen and CO₂ are taken up according to the reaction



and no oxygen is given off. It appears that anaerobic conditions and the presence of hydrogen result in the adaptive production or activation of hydrogenase; if hydrogen is replaced by nitrogen, hydrogenlyase appears, liberating hydrogen from endogenous substrates. If the intensity of the illumination is increased above a certain value the cell reverts to its original mode of photosynthesis and oxygen is again given off and hydrogenase activity ceases. The plant and bacterial photosynthetic reactions can be distinguished by their behaviour with inhibitors (see Table 5) and also

TABLE 5

Poison	Type of Photosynthesis	
	Bacterial	Plant
Cyanide 10 ⁻⁴ M . .	Inhibits	Inhibits
Hydroxylamine 10 ⁻⁴ M . .	No effect	Inhibits
2, 4-Dinitrophenol . .	Inhibits	Inhibits
Carbon monoxide . .	Inhibits	No effects

by the fact that the plant reaction is highly endergonic whilst the bacterial overall reaction occurs with little or no energy change. Since both reactions depend on the energy derived from light it seems probable that the photosynthetic reaction is the same in both

¹ Roedelien, 1935.² Gaffron, 1933.³ Nakamura, 1937.⁴ Gaffron, 1940.

cases ; this may be the formation of a $\text{CO}_2 - \text{H}_2\text{O}$ complex which splits into a reduced compound leading to carbohydrate formation and an oxidised product of a peroxide character. In bacterial photosynthesis the peroxides are reduced by hydrogen donators (H_2S , organic compounds or H_2), catalysed by their appropriate enzymes. At the higher intensities of illumination characteristic of plant photosynthesis the peroxides are decomposed to water and oxygen, probably through the action of an additional enzyme characteristic of the plant system. It thus seems possible that the bacterial mode of photosynthesis is the more primitive and the adoption by the green plant of an additional mechanism for decomposing the peroxide complex delivers it from the necessity for maintaining anaerobic conditions and enables it to invade dry land.

From the experiments just described it is now clear that in the photosynthetic bacteria we have a remarkable group of organisms depending for their carbon supply on carbon dioxide and for their energy on solar radiation ; in these respects their type of metabolism resembles the green plant. Here, however, the resemblance ends, for whereas the photosynthetic plant employs a mechanism in which water appears as the reducing agent liberating oxygen (equation 1), these bacteria use either hydrogen sulphide liberating free sulphur (equation 2), or inorganic sulphur compounds and free sulphur forming sulphate (equations 3 and 4) or organic hydrogen donators with which free hydrogen may be included (equations 5 and 6, Table 6). The use of solar energy relates

TABLE 6

Organism	Reducing agent	Characteristic reaction
Green plant .	H_2O	$\text{CO}_2 + \text{H}_2\text{O} = \text{CH}_2\text{O} + \text{O}_2$. (1)
<i>Thiorhodaceæ</i>		
(a) Green sulphur bacteria .	H_2S	$\text{CO}_2 + 2\text{H}_2\text{S} = \text{CH}_2\text{O} + \text{H}_2\text{O} + 2\text{S}$ (2)
(b) Purple sulphur bacteria .	H_2S	$\text{CO}_2 + 2\text{H}_2\text{S} = \text{CH}_2\text{O} + \text{H}_2\text{O} + 2\text{S}$ (3)
	S	$3\text{CO}_2 + 2\text{S} + 8\text{H}_2\text{O} = 3\text{CH}_2\text{O} + 3\text{H}_2\text{O} + 2\text{H}_2\text{SO}_4$ (4)
<i>Athiorhodaceæ</i>		
(c) Purple bacteria	Organic acids, etc., and hydrogen	$\text{CO}_2 + \text{C}_4\text{H}_8\text{O}_2 + \text{H}_2\text{O} = 5\text{CH}_2\text{O}$ (5) $\text{CO}_2 + 2\text{H}_2 = \text{CH}_2\text{O} + \text{H}_2\text{O}$. (6)

these organisms to the green plant but the failure to use H_2C as the hydrogen donator sharply differentiates them. The use of inorganic substances as oxidisable material relates the *Thiorhodaceæ* to the chemosynthetic organisms, from which they are sharply differentiated by their anaerobic mode of life and use of solar energy. The use of organic hydrogen donators and

molecular hydrogen relates both types of purple bacteria to heterotrophic organisms, from which they are differentiated by their use of solar energy and the almost complete transformation of the carbon of the substrate into cell material. The question whether the purple sulphur bacteria (*Thiorhodaceæ*) can also use organic hydrogen donors has been a matter of controversy. Gaffron believed that he showed that in the cases where it appeared to occur two reactions were taking place, viz. a reduction of sulphate to sulphide in the dark and a reduction of CO_2 by sulphide in the light. After some polemics it now appears that the reduction of hydrogen sulphide in the dark is due to a reduction of intracellular sulphur by unknown hydrogen donors and that the amount so formed is quantitatively negligible compared to the amount of carbon dioxide subsequently reduced.¹

The bacterial pigments

Spectroscopic examination of various members of the *Thiorhodaceæ* and *Athiorhodaceæ* reveals the presence of strong absorption bands in the infra-red due to bacterio-chlorophyll, the evidence for which is supplied by French's study of the photosynthetic action spectrum.² The same worker showed also that rupture of the cells gave a preparation with the same spectrum but one which was photosynthetically inactive. Extraction with ethanol gave a solution with a band shifted nearer the visible end of the spectrum; alcoholic extracts of all members of purple bacteria so far examined show the same band at 774 $\text{m}\mu$, suggesting that the chlorophyll is identical in the different species. The fact that in whole cells the position of the bands in the infra-red varies is probably attributable to the different proteins to which it is attached (see Table 7).^{3, 4} These observations refute the earlier view

TABLE 7⁵

INFRA-RED ABSORPTION BANDS OF VARIOUS SPECIES OF PURPLE BACTERIA

Strain	Absorption maxima $\text{m}\mu$						
<i>Thiorhodaceæ</i>							
Type 1 . . .	895	—	—	855-850	—	796	—
Type 2 . . .	895	—	865	—	804	—	—
<i>Athiorhodaceæ</i>							
Type 1 . . .	—	892-895	—	850	—	799	—
Type 2 . . .	—	—	880-863	—	802	—	—
Type 3 . . .	—	—	875	—	—	800	—
Alcoholic extract of all types . . .	—	—	—	—	—	—	774

¹ van Niel, 1936.² French, 1937.³ Wassink *et al.*, 1939.⁴ Katz & Wassink, 1939.⁵ Wassink *et al.*, 1939, *Enzymol.*, 7, 113.

advanced by Schneider¹ that two bacterio-chlorophylls exist side by side in the purple bacteria analogous to chlorophylls *a* and *b* of the green plant, and are in agreement with Fischer's^{2, 3} work on bacterio-chlorophyll suggesting its close relationship to chlorophyll *a* of the green plant. Little information is available on the chlorophyll of the green sulphur bacteria. Studies on the carotenoids of the purple bacteria^{4, 5} have resulted in the characterisation of spirilloxanthine having the empirical formula $C_{48}H_{66}O_3$ and containing 15 double bonds per molecule.

¹ Schneider, 1934.

² Fischer & Hasenkamp, 1935.

³ Fischer & Lambrecht 1937.

⁴ van Niel & Smith, 1935.

⁵ van Niel, 1944.

CHAPTER XI

ENZYME VARIATION AND ADAPTATION

THE fact that bacteria have in the course of evolution developed in comparison with other groups such an immense variety of chemical mechanisms is in itself an indication of their great potential variability. This is indeed their most marked characteristic and shows itself at several levels—in species and strain variation, and during the life of each cell in response to changes in internal and external environment.

Adaptation by natural selection acting on a mutant arising spontaneously

An example of this type of variation was first noted by Massini¹ and consists in the occurrence in *Esch. coli* of a mutant able to ferment galactose. Actually it has been later claimed that the mutation consists in permeability of the cell towards galactose rather than in an enzyme change,² but the actual nature of the mutation is a less important consideration than the manner of its origin and stabilisation.

It was first observed³ that when a certain strain of *coli* was plated on "Endo" medium (broth agar containing lactose and coloured with fuchsin) the colonies first developing were white, showing that lactose was not fermented. After some days red lactose-fermenting secondary colonies developed as knobs on the top of the white colonies. If subcultures were taken from the white colonies on to Endo plates the phenomenon was repeated, but if taken from the red knobs red colonies developed from the start but no white ones. The culture from the secondary colonies ferments lactose for many generations and in some cases has not been known to revert, though cases are recorded when the culture kept on lactose-free agar reverted to the original type.^{4, 5} Many examples of variations of this sort are recorded—as, for example, the appearance of saccharose fermentation (due doubtless to invertase) in a *coli*-like organism⁶ and rhamnose-fermenting secondary colonies in *Bact. typhosum*.⁷ Massini believed that the appearance of lactose-fermenting secondary colonies was due to a mutation

¹ Massini, 1907.

² Deere, 1939.

³ Massini, 1907.

⁴ Baerlein, 1912.

⁵ Hershey & Bronfenbrenner, 1937, 1938.

⁶ Burri, 1910.

⁷ Reiner Muller, 1909.

and called his strain *Bact. coli mutabile*. The mode of origin of such strains has been shown by Lewis¹ to be due to natural selection working on a spontaneous variation in the culture. Thus, working with a strain of *Bact. coli mutabile*, he showed that it would grow on synthetic medium with lactose as sole source of carbon; the growth as compared with that on peptone was slow at first but attained about the same maximum. When growth on peptone and on lactose peptone was compared, growth was parallel for 24 hours, after which it became suddenly much more rapid on the lactose medium. The question Lewis set out to answer was whether a *mutabile* strain grown in the absence of lactose contains variant cells capable of attacking that sugar, or whether such variant cells are produced only in response to the presence of the sugar. For this investigation a culture from an agar slant was diluted serially 1/10 to 1/10⁹. Dilutions were plated on lactose synthetic medium and on to control plates of glucose synthetic medium; the highest dilutions were plated also on to broth agar to obtain counts. The colonies on glucose synthetic agar and broth agar were approximately equal in number. On lactose synthetic agar colonies were obtained only from the low dilutions, showing that approximately only 1 cell in 10⁵ could grow on this medium. The colonies so obtained were indistinguishable from those obtained by plating secondary colonies from the ordinary culture on lactose broth, that is, they were the variants. The variants therefore form approximately 1 in 10⁵ in an ordinary culture without lactose. Lewis also showed that all colonies on plain agar contain variants in about the same proportion (Table 1).

TABLE 1

Colony	Numbers obtained by plating on		$\frac{B}{A}$
	A Lactate synthetic agar	B Beef extract agar	
1	6300	3540×10^6	5.62×10^5
2	3300	1610×10^6	4.87×10^5
3	2900	730×10^6	2.4×10^5
4	2200	840×10^6	3.8×10^5
5	2080	1200×10^6	5.7×10^5

This was shown by taking five colonies from broth agar and determining the relative number of cells able to grow on lactose synthetic agar and broth agar respectively. Table 1 shows that the five colonies all contained mutants in remarkably constant proportions. The phenomenon of *mutabile* strains giving rise to second-

¹ Lewis, 1934.

dary colonies is explicable on the ground that ordinary cultures contain a constant low proportion of mutants. When plated on to broth agar containing the sugar in respect of which the culture mutates, the variants are so few that they are not present in sufficient numbers to characterise the colonies until these have ceased growing owing to the exhaustion of the broth medium. When this has occurred the mutants alone are able to multiply in virtue of the sugar present and the secondary colony then appears. This is a true case of adaptation due to natural selection.

Another example of spontaneous mutation, this time in the direction of gaining a synthetic ability, is reported by Monod.¹ An apparently normal strain of a coliform organism grew well in broth and also in synthetic medium (S) in which glucose provided the carbon and ammonia the nitrogen. When plated on to washed agar and synthetic medium (GS) two kinds of colony appeared, type N, microscopic (0.1 mm. diameter), transparent, filamentous and very numerous, and type M, large (1.0–1.5 mm. diameter), hyaline and non-filamentous. On subculturing from N on to GS plates both types of colony always appeared in about the same relative proportion. On growing N in liquid S medium the N type died out after several passages, and on replating on GS medium M colonies only appeared; the M type remained unchanged after repeated subcultivation in broth. Nutritional studies on GS plates with single amino-acids revealed that the N colonies consisted of cells which had lost the power of synthesising methionine whilst the M colonies grew equally well in its presence or absence. Hence N colonies subcultivated on to GS plates and methionine gave rise only to large colonies, M being then at no advantage over N. If N strain was grown for 10 or more subcultivations on S plus methionine and then plated on GS the original picture—numerous microscopic and few large colonies—reappeared.

Thus the original culture consists of cells unable to synthesise methionine; these give rise to mutants able to dispense with and therefore to synthesise this amino-acid. Special plate counts on GS with methionine and glutamic acid revealed that the ratio of M/N in the culture was about 1/2500. Further experiments on the rate of growth of the two types in the same medium showed that in the log. phase it was 1.45 and 1.05 generations per hour for N and M respectively, from which data it was calculated that the rate of mutation was $1/10^4$.

The spontaneous variation of organisms in the loss of enzymes at the root of the development of strains with loss of synthetic powers in environments where essential nutrients or building materials are in good supply. It is necessary, however, to postulate

¹ Monod, 1946, in the press.

some advantage to the organism to make use of this or that ready-made compound rather than to continue to synthesise it, otherwise the nutritionally non-exacting strain would continue side by side with the new exacting strain. It is possible that mutants, forced by the loss of an enzyme to use a molecule lying in the environment, complete their growths more rapidly and so outgrow the more synthetically capable parent strain. This view is corroborated by nutritional studies where organisms are able to synthesise a given growth factor or vitamin yet grow more rapidly when it is supplied. It is implicit in general experience that growth of non-exacting organisms on synthetic media is much slower than on rich media. Actually this was the case with the methionine mutant reported above, where the exacting strain N multiplied more rapidly than the non-exacting strain M.

Artificial production of mutants

An interesting and very informative line of work has been for some time in progress on the production of artificial mutants of the bread mould *Neurospora crassa*. This is strictly outside the scope of this book but its implications are so wide that a brief reference must be made to it. The approach to the problem is nutritional but the results are of general biochemical and genetical importance. The parent strain of *Neurospora* used maintains growth and reproduction on a synthetic medium with one source of carbon, biotin being the only additional factor required. The parent culture is exposed to X-rays or U-V light and plated out on a fully nutrient medium with yeast extract. The resulting colonies are subcultured into a fully nutrient medium and into the original synthetic medium. A certain small proportion (the mutants) fails to grow in the latter and on examination this is found to be due to failure to synthesise some single growth factor or amino-acid. For example, a U-V-induced mutant of *N. crassa* was found to be exacting towards leucine ("leucineless" strain). This strain was mated with the normal type and produced the usual eight sexual spores. The differentiating characteristics of the two types "leucine +" and "leucine -" segregated in the offspring, four of which were able and four unable to synthesise leucine. This organism, being heterothallic and sexual, supplies the information that the synthetic power by which the parent and mutant differ is due to the presence or absence of one gene.¹ Further study reveals the fact that the failure of each mutant to synthesise a given product may be due to the failure of one step only in the synthesis, i.e. to the absence of one enzyme. This leads to the conclusion that one gene controls the formation of one enzyme.

¹ Regnery, 1944.

Whether this conclusion is valid is not for the author to say, but a further interesting piece of evidence may be quoted. By the usual irradiation methods seven strains of *Neurospora* were obtained all exacting to arginine. These were of three kinds: mutant 1, able to replace arginine by either ornithine or citrulline;

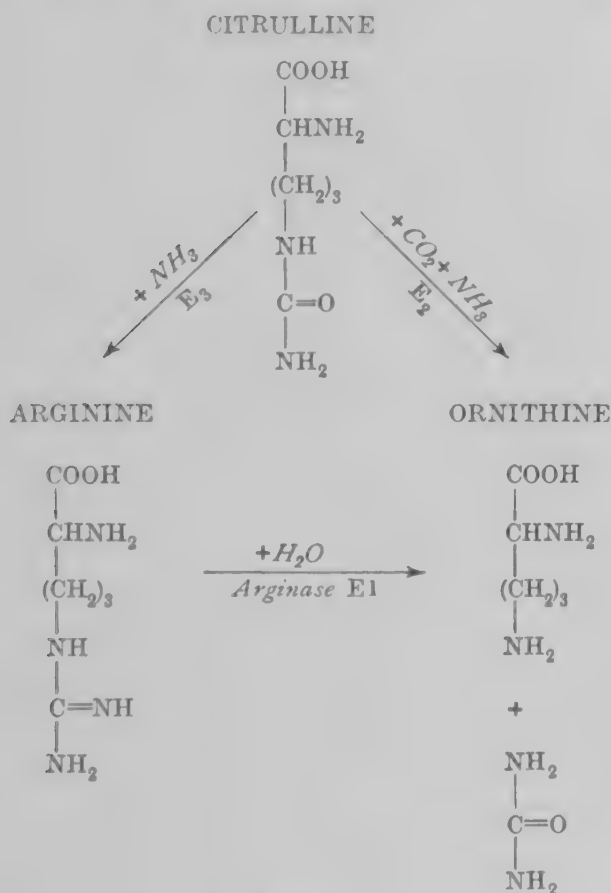


FIG. 1

mutant 2, able to replace arginine by citrulline but not by ornithine;¹ and mutant 3, unable to replace arginine either by ornithine or citrulline. The replaceability of arginine by either citrulline or ornithine proves that the organism builds arginine via the Krebs cycle (Fig. 1).

Mutant 1 can use ornithine or citrulline and therefore has enzymes 2 and 3; mutant 2 can use citrulline but not ornithine and has therefore lost enzyme 2; mutant 3 can use neither citrulline or ornithine and has therefore lost enzyme 3 (and possibly 2 also). The evidence is therefore in favour of ornithine and citrulline being

¹ Srb & Horowitz, 1944.

intermediates in the synthesis of arginine and of one mutant arising by loss of one gene which controls one enzyme. Evidence on the synthesis of tryptophan was mentioned in Chapter V, p. 133.

Another method by which mutants with loss of one enzyme can be produced artificially consists in growing the organism in sublethal amounts of an inhibitor for the enzyme concerned.

Cyanide yeasts

Several observers have grown yeasts in the presence of cyanide, and have reported a decreased oxidative capacity and sensitivity to cyanide, also the abolition of the Pasteur effect. A permanent and profound change due to growing *S. cerevisiae* (Hansen) once only in $M/1000$ KCN is reported by Stier and Castor.² The cyanide yeast showed a decreased respiration on glucose and an increased anaerobic glycolysis as compared with the parent strain. The cytochrome oxidase test was negative with *p*-phenylene diamine and the effect on the respiration of KCN and NaN_3 was negative. The cytochrome bands no longer disappeared in oxygen. All these observations indicate the disappearance of cytochrome oxidase; cytochrome C bands and catalase were still present. In addition to the abolition of cytochrome oxidase an alternative oxidative mechanism insensitive to $-\text{CN}$ and NaN_3 was developed or increased; this may be explicable by the increased flavin in cyanide yeast noted by Pett.³

A sulphapyridine-induced mutation of pneumococcus type I

An effect analogous to that of $-\text{CN}$ on yeast has been produced by sulphapyridine on the pneumococcus. A drug-resistant strain tolerating $1/16,000$ sulphapyridine was obtained by repeated subcultivation in increasing concentrations of the drug. The resistant strain (RS) differed from the parent strain (PS) in (1) producing relatively very little H_2O_2 aerobically; (2) in the loss of dehydrogenases for glycerol, lactate and acetaldehyde, that for glucose remaining unimpaired. In washed suspensions of the PS these same dehydrogenases are suppressed or greatly inhibited by sulphapyridine, whilst glucose dehydrogenase is unaffected. It seems that here also growing a cell in the presence of a poison results in the loss of those enzymes for which the poison acts as an inhibitor.

A variant on this effect was obtained by growing *Esch. coli* in the presence of a high concentration of sulphanilamide (2×10^{-2}) to which it had been trained, and methionine, which antagonises this drug. After 30 transfers a strain permanently exacting to

¹ Pett, 1936.

² Stier & Castor, 1941.

³ Pett, 1936.

methionine was obtained; cultivation of controls in sulphanilamide without methionine did not have this effect. The tentative explanation put forward by the authors was that sulphanilamide interferes in some way with methionine synthesis and that in the absence of methionine the cell diverts the course of its metabolism so as to protect the synthesis; in the presence of methionine this adjustment is unnecessary and does not take place, with the result that the cell becomes permanently dependent on the methionine of the medium.¹

Effect of iron-deficient media on enzyme production

Closely connected with the cyanide effect is the result of iron deficiency. The media were rendered iron-deficient by treatment with 8-hydroxyquinoline and chloroform and then contained 0.0007-0.003 $\mu\text{g. ml.}$ of iron.² Organisms having incomplete cytochrome systems (*coli*, *pneumoniae* and *Aerobacter aerogenes*) required 0.02-0.03 $\mu\text{g. ml.}$ Fe for optimum growth, whilst *Ps. aeruginosa*, with a 4-band cytochrome system and vigorous catalase and peroxidase, required 3-4 times that amount.³

Using *Aer. indologenes* a comparison between cells grown in iron-deficient media and in optimal iron media showed very marked changes; the iron-deficient cells had only 5% of the normal activity of catalase and peroxidase and the cytochrome bands at 560 $m\mu$ and 590 $m\mu$ were invisible. In agreement with these changes the Q_{O_2} of the aerobically grown deficient cells on formate, acetate, pyruvate and lactate was much reduced, but the Q_{O_2} on glucose was unchanged. The iron-deficient cells grown anaerobically showed the same effect except that the Q_{O_2} glucose of the iron-deficient cells was higher than normal. The effect of -CN on both types of cell caused the same proportionate decrease in respiration (see Tables 2 and 3). The disappearance of hydrogenase, formic dehydrogenase and formic hydrogenlyase in iron-deficient cells has been referred to earlier (p. 81). No claim was made that the changes in iron-deficient cells were permanent.

The dehydrogenases (as measured by MB) of deficient organisms showed decreased activity in the case of malate and succinate; the decrease was about 50% in both cases. Formic dehydrogenase had nearly disappeared and hydrogenase was not detectable; the dehydrogenases of glucose, lactate and ethanol were unaffected.⁴

The removal of iron from the medium profoundly alters the fermentation of *CL. welchii*.⁵ The normal fermentation products

¹ Kohn & Harris, 1942.

² *Ibid.*, 1944.

⁴ *Ibid.*

² Waring & Werkman, 1942.

⁵ Pappenheimer & Shaskan, 1944.

TABLE 2¹

Culture	Q ₀₂				
	Glucose	Lactate	Pyruvate	Acetate	Formate
<i>Aer. indologenes</i>					
Grown aerobically:					
Normal 1 . . .	73	38	41	13	60
Normal 2 . . .	51	—	53	8	81
Deficient 1 . . .	55	11	17	0	1
Deficient 2 . . .	68	—	13	1	1
Grown anaerobically:					
Normal 3 . . .	34	27	36	3	36
Normal 4 . . .	34	—	36	—	57
Deficient 3 . . .	49	15	13	1	10
Deficient 4 . . .	56	12	18	1	11

TABLE 3²EFFECT OF 0.001 MCN ON Q₀₂

Culture	Glucose	Lactate	Pyruvate	Acetate	Formate
<i>Aer. indologenes</i>					
Normal 1 . . .	73	38	41	15	60
Normal 1 + CN . . .	9	3	6	2	—
Deficient 1 . . .	55	11	17	—	1
Deficient 1 + CN . . .	4	—	3	—	—

of this organism are lactic, acetic and traces of butyric acid, ethanol, CO₂ and H₂. Growth on a medium of low iron content decreases all the products except lactic acid, which then forms the main fermentation product and may amount to 1.73 mols./mol. glucose (see Table 4).

TABLE 4³

	Media	
	Low Fe. 0.4 mg./l.	High Fe. 1.25 mg./l.
Fe/mg. bacterial N. (mg.) . . .	0.0005	0.0039
Glucose fermented mM . . .	1.00	1.00
Lactic acid mM . . .	1.75	0.42
Total vol. acids mM . . .	0.06	0.88
Ethanol mM . . .	0.025	0.16
CO ₂ mM . . .	0.33	1.35
H ₂ mM . . .	0.38	1.93
Carbon recovery, % . . .	95.5	89.2

It has also been shown with another strain of *Cl. welchii* that fermentations carried out in an atmosphere of CO result in com-

¹ Waring & Werkman, 1944.³ Pappenheimer & Shaskan, 1944.² Ibid.

plete inhibition of gas production and that lactic acid then forms 70% of the fermentation products; this effect is not reversed by light at intensities sufficient to reverse CO poisoning in the case of yeast.¹

Induced sulphonamide resistance

It has already been mentioned that one of the most favoured explanations for sulphonamide action is its interference with the production of the essential growth factor *p*-aminobenzoic acid. Artificially induced sulphonamide resistance *in vivo* is accompanied by the production in the resistant strain of greatly increased amounts of *p*-aminobenzoic acid. Thus a strain of *Staphylococcus* was rendered sulphonamide-resistant by repeated subculturing in increasing amounts of sulphonamide. After 130 subcultures the organism produced maximal growth in the presence of 2 mg./ml. sulphapyridine, 1 mg./ml. sulphadiazine and 0.5 mg. ml. sulphathiazole.² A resistant strain produced in this way was found to produce 70 times as much *p*-aminobenzoic acid as the parent strain, and the induced change was reported to be permanent.³

Mechanism controlling polysaccharide production in type III pneumococcus

The spontaneous dissociation of bacterial species into two strains with rough and smooth colonies has been shown in many cases to be due to the sudden loss of the power to form capsules, such organisms giving rise to rough colonies (R) whilst the smooth (S) forms retain the polysaccharide capsule of the parent strain.

It was shown by Griffith⁴ that if a small inoculation of living organism of R-nonencapsulated type II pneumococcus is injected into the subcutaneous tissues of the mouse, along with a heavy inoculum of heat-killed type I strain, the organism isolated is an S form carrying the type I capsule. The same result was achieved *in vitro*, showing that the living tissues of the mouse had no part in the change-over.⁵ A further simplification was achieved when the intact heat-killed cells were replaced by extracts of cells disrupted by desoxycholate.⁶ Twelve years later Avery, McLeod and McCarty used as a transforming agent a polymer of desoxyribose-nucleic acid prepared from mass cultures (50-75 l.) of type III pneumococcus. The substance was obtained in a high degree of purity by disintegration of the cells, removal of polysaccharide and

¹ Bacon, private communication.

³ Landy *et al.*, 1943.

⁵ Dawson & Sia, 1931, and Sia & Dawson, 1931.

⁶ Alloway, 1932, 1933.

² Vivino & Spink, 1942.

⁴ Griffith, 1928.

protein and repeated precipitations with alcohol. The final product was identified as a polymer of desoxyribosenucleic acid by elementary analysis, action of the specific enzyme desoxyribose-nuclease, homogeneity in the ultra-centrifuge and the presence of a single substance of high electrophoretic mobility. This substance when added to a small culture of type II R pneumococcus with heated serum effected the transformation to pneumococcus with the type III capsule. The nucleic acid polymer was active in a concentration of 0.009–0.0012 $\mu\text{g.}/\text{ml.}$ ¹

It thus appears that a cell which has lost its power to synthesise a capsular polysaccharide can gain the power to synthesise a different one by growing in the presence of a highly purified polymer of desoxyribosenucleic acid obtained from the homologous organism. We do not, of course, know whether the initial loss involved in the change from S to R is due to the loss of a single enzyme in the chain of those necessary to synthesise the capsular material, or whether it involves the loss of the whole series of enzymes. The importance of the observation is that it carries irrefutable proof that nucleic acid controls enzyme production, a fact towards which converging evidence already pointed. It also shows that in carefully controlled conditions a dividing cell can use a portion of nucleic acid polymer from a related organism to control the synthesis of a substance alien to itself and characteristic of the cell from which the nucleic acid was derived.

Probably such interference could only be achieved with organisms whose nuclear material was loosely organised, but in any case it is a piece of experimental genetics whose significance it would be difficult to overrate.

Temporary change in enzyme activity due to changes in growth medium

This type of adaptation is a direct response of the enzymic composition of the cell to the constituents of the growth medium. It is definitely temporary and does not affect the heredity mechanism of the cell, which reverts to normal (if indeed a "normal" bacterial cell exists) when the organism is grown without the specific stimulus. The early literature contains many references to this type of variation but the clear recognition of the phenomenon is due to a thesis by Karström,² and may be exemplified from one of many experiments recorded in his paper.

A xylose-fermenting strain of *Bact. aerogenes* was grown on whey and the washed suspension of the organism added to xylose in the presence of chalk; no fermentation occurred in 15 hours. A source of nitrogen was then added to the tubes (yeast water or

¹ Avery *et al.*, 1944.

² Karström, 1930.

ammonium sulphate) and fermentation set in 2 hours later. When the organism was grown in xylose broth and treated as before, fermentation set in immediately, whilst the fermentation of glucose as opposed to xylose occurred whether glucose was present in the growth medium or not. The glucose-fermenting enzyme, being apparently a constant constituent of the cell, is called "constitutive," whilst the xylose-fermenting enzyme is "adaptive."

Table 5 shows the relation of constitutive and adaptive enzymes

TABLE 5¹
FERMENTATION BY *Betacoccus arabinosaceus*

Grown in	Glucose	Fructose	Sugars subsequently fermented					
			Mannose	Galactose	Arabinose	Sucrose	Maltose	Lactose
Glucose, 2% . . .	+	+	+	o	o	o	o	o
Sucrose, 2% . . .	+	+	—	o	o	+	o	o
Maltose, 2% . . .	+	+	—	o	o	+	+	o
Lactose, 1% . . .	+	+	—	+	o	+	o	+
Galactose, 0.8% . .	+	+	—	+	o	+	o	o
Arabinose, 0.8% . .	+	+	+	—	+	—	—	—
Carbohydrate-free medium . . .	+	+	+	—	—	+	+	o

o = not fermented.

— = absence of data.

TABLE 6²
FERMENTATIONS BY *Bact. coli*

Grown in	Sugars subsequently fermented				
	Glucose	Sucrose	Raffinose	Maltose	Lactose
Sucrose, 3% . . .	+	+	o	o	o
Maltose, 1% . . .	+	+	—	+	o
Lactose, 1% . . .	—	o	—	+	+

Grown in	Sugar subsequently fermented			
	Xylose	Arabinose	Rhamnose	Glucose
Arabinose . . .	o	+	o	+
Xylose . . .	+	o	o	+

o = not fermented.

— = absence of data.

¹ Karström, 1930.

² Ibid.

in *Betacoccus arabinosaceus*. Here lactase, galactozymase and arabinozymase are adaptive and glucozymase and invertase constitutive; the case of maltase will be referred to later. With *Lactobacillus pentoaceticus* (*Betabacterium* 20) glucozymase is adaptive and arabinozymase constitutive. *Bact. coli* I showed the relations given in Table 6.

Although we have followed Karström in his classification of enzymes into constitutive and adaptive, the distinction between the two is not quite clear-cut, as he himself was the first to point out. Thus in the case of invertase in dried preparations of *Bact.*

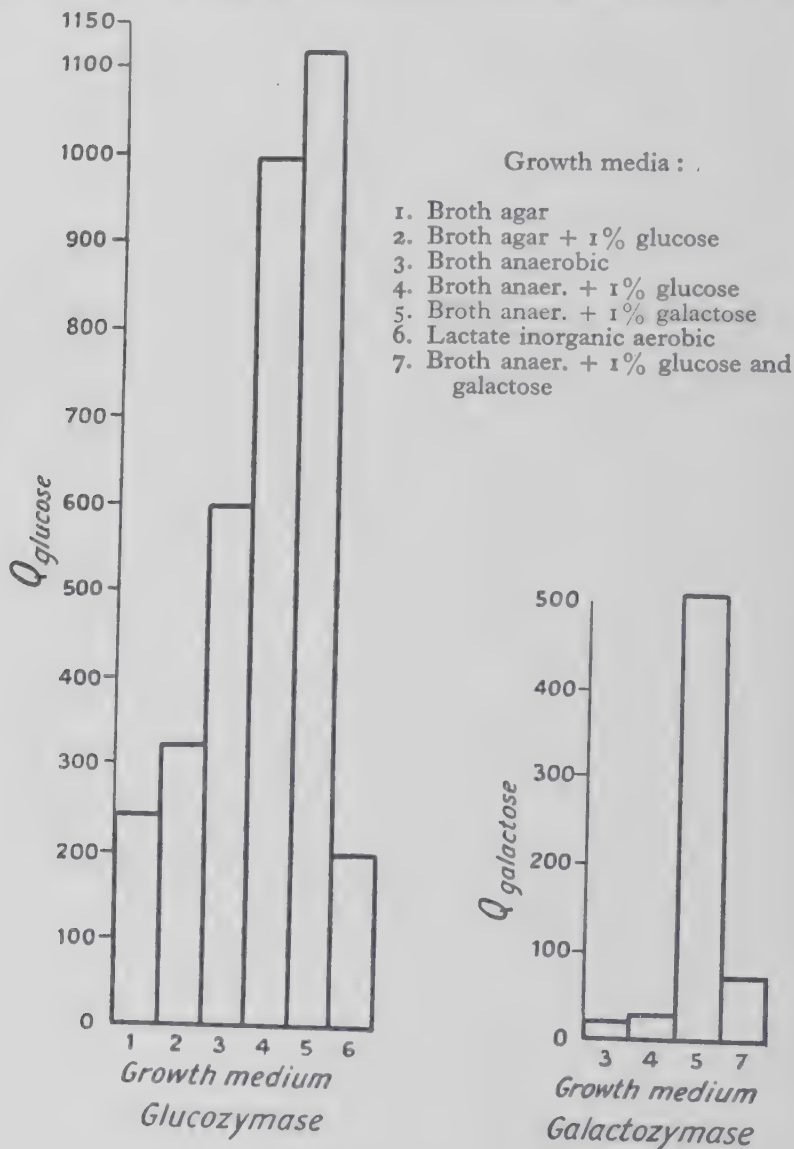


FIG. 2

and the enzyme is present on whatever substrate the organism is grown, but much increased when sucrose is present.¹ In the case of *Bact. coli* glucozymase is regarded as a constitutive enzyme, but it is nevertheless subject to fluctuations due to the growth medium. Fig. 2² shows that it is influenced both by conditions of aeration and by the addition of glucose or galactose. The two hexoses have an approximately equal stimulating effect on the enzyme production. Even in the case of galactozymase, which is classed as an adaptive enzyme, a slight fermentation of galactose occurs in the absence of substrate in the growth medium, but the effect of adding galactose is relatively much greater than in the case of glucose.

Teleological considerations

Since Karström's paper the distinction between constitutive and adaptive enzymes has received considerable attention. It has become clear that the former comprise only a small proportion of the enzymic constituents of the cell and that these for the most part operate the breakdown of substances such as glucose, lactate, etc., most commonly found in the natural environments. Even so the quantity or activity of most constitutive enzymes is greatly increased when the cell is grown in conditions which oblige it to depend on the specific substrate for its growth. Enzymes attacking the less common substrates such as the rarer polysaccharides and sugars, chitin, creatine, citric acid, etc., do not form a permanent part of the cell. When the specific substrate appears in the environment of the growing organism it exerts some influence on the developing enzyme proteins, resulting in the production of the enzyme able to adsorb and decompose it. Even so every bacterium cannot adapt to every naturally occurring compound, which implies that different organisms carry certain basal proteins which can be modified during growth to form special groups of enzymes. The technique of selective media by which large numbers of soil organisms are placed in an environment where growth depends on the development of an enzyme able to initiate the attack on a particular compound is an application of this conception. The number of naturally occurring organic compounds of plant and animal origin is vast and the size of a microbe limited; it is therefore not surprising that the adaptive mechanism for the whole range is not contained within the limits of any one cell.

The mass action theory of enzyme formation

So far no mechanism has been put forward to explain the production of chemical adaptation. Yudkin³ has, however, suggested

¹ Karström, 1930.

² Stephenson & Gale, 1937 (1).

³ Yudkin, 1938.

that all cases of apparent enzyme production, resulting immediately from the presence of the substrate in the growth medium, are actually cases of enzyme increase, the amount present in the absence of substrate being so small that it may sometimes elude measurement; this has since been proved in many cases. Supposing that every enzyme is directly formed from a precursor which normally forms some part of the cell protoplasm, there must exist an equilibrium between the precursor and the enzyme. On the addition of substrate combination takes place between it and the enzyme and more enzyme would be formed from the precursor in order to restore the equilibrium. Yudkin points out that cases where enzyme production is increased by the products of enzyme action as well as by the substrate itself are in support of this theory, since in the cases where enzyme action can be shown to be reversible this is due to the combination between enzyme and the products of the reaction, and such a combination should affect enzyme production from the precursor just as does the combination between enzyme and substrate. Instances of this kind are the reported stimulation of diastase in *A. niger* by maltose as well as by starch,¹ and the increase of yeast invertase by fructose and glucose as well as by sucrose.^{2*} More recently it has been shown that an organism which becomes adapted to the oxidation of *p*-aminobenzoic acid by growth on that compound is equally well adapted by the presence of the *N*-acetyl derivative and by *p*-nitrobenzoic acid; the former was deacetylated prior to oxidation by both adapted and non-adapted cells, but subsequently oxidised only by the latter. A much slighter but still definite adaptive effect towards *p*-aminobenzoic acid was obtained with the glycyl derivative and with the methyl ester and with *p*-toluic acid, but not with *m*-aminobenzoic acid. The *o*-isomer (anthranilic acid) does not stimulate the production of the *p*-enzyme but produces the *m*-enzyme. It is interesting that the organism when grown in tryptophan produces the oxidative enzyme for anthranilic acid to the same extent as when grown on anthranilic acid itself. On the other hand the production of the *p*-enzymes is not stimulated by growth on the sulphonamides and sulphapyridine is not attacked by the organism adapted to *p*-aminobenzoic acid, nor does it prevent the oxidation of the latter by the adapted cells.³

The relation between adaptive enzyme formation and growth

In general, adaptation in the Karström sense is correlated with cell multiplication, but this is not an invariable rule. Dienert⁴

¹ Funke, 1923.

² Euler & Cramer, 1913.

* For more recent views see Spiegelmann, 1946.

³ Mirick, 1943.

⁴ Dienert, 1900.

first reported the production of galactozymase without growth and his observation was corroborated and extended by following the production of the enzyme by cells in galactose and buffer, but with no source of nitrogen; simultaneous cell counts showed that the appearance of the enzyme occurred whilst the cell numbers remained stationary.¹

Similar observations have been made in the case of formic hydrogenlyase,² tetrathionase³ and several sugars.⁴

Although it is now established that adaptation can occur in the absence of cell multiplication, so far it has not been found to occur in the presence of growth inhibitors or cell poisons;⁵ nevertheless there is a suggestion in cells poisoned with U-V light that adaptation may occur in cells incapable of multiplication, i.e. that in partially poisoned cells power to adapt may survive power to reproduce.⁶

Some further light on this problem has been supplied by the adaptive enzyme tetrathionase, by which tetrathionate is reduced to thiosulphate in the presence of a suitable hydrogen donator, e.g. mannitol, according to the equation



Incubation with both tetrathionate and the donator is necessary

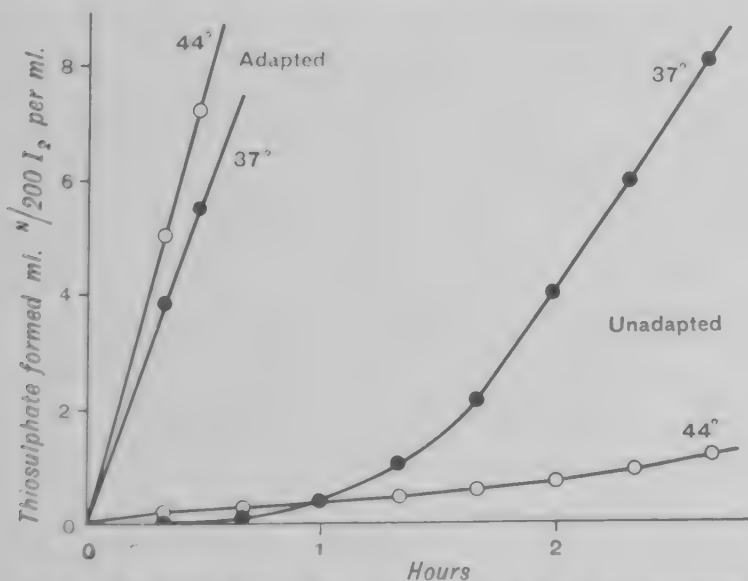


FIG. 3. — Comparison of effects of temperature on tetrathionate reduction by unadapted and by adapted suspensions of *Bact. paratyphosum* B⁷

¹ Stephenson & Yudkin, 1936.

² Knox & Pollock, 1944.

³ Stephenson & Yudkin, 1936.

⁴ Pollock, *British J. exp. Path.*, 26 (1945).

⁵ Stephenson & Stickland, 1933 (1).

⁶ Hegarty, 1938.

⁷ Ibid.

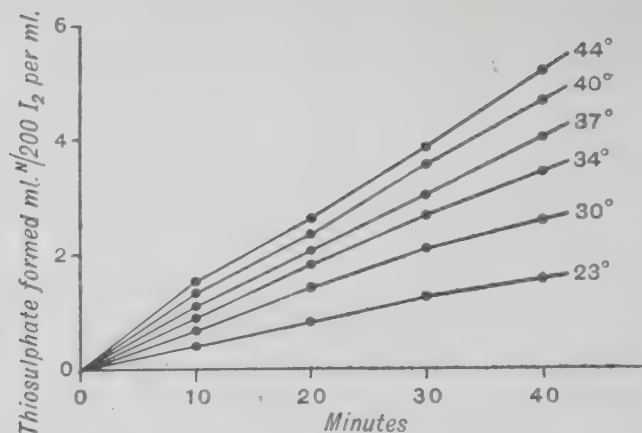


FIG. 4.—Effect of temperature on tetrathionate reduction by previously adapted suspensions of *Bact. paratyphosum B*¹

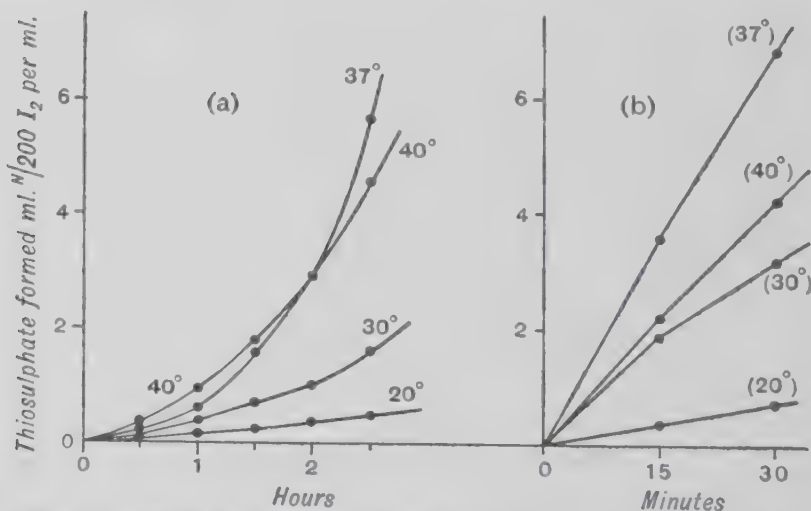


FIG. 5.—Proportions of active enzyme present in suspensions of *Bact. paratyphosum B* after adaptation for 2½ hours at different temperatures³

(a) Tetrathionate reduction during adaptation at different temperatures.

(b) Tetrathionate reduction at 44° after adaptation for 2½ hours as in (a). (Temperatures in brackets are those of previous adaptations.)

for full adaptation to occur, and it has been shown that this can take place without measurable increase in cell numbers or cell nitrogen or dry weight.³ Adaptation in this case takes about 100 minutes, after which the rate of reduction is linear with time and equal to the rate obtained starting with fully adapted cells.

The optimal temperature for reduction is 44° but adaptation at this temperature is very slow, the optimal temperature for the

latter being 36–37°, which is approximately that for cell growth (Figs. 3, 4 and 5).

We may perhaps consider that adaptation or enzyme formation is a change in cell proteins governed itself by an enzyme A, the latter being a constitutive component of the cell present in minimal amounts in all cells of the strain. In most circumstances A remains combined or in equilibrium with a second protein or enzyme B forming the complex AB, which on dissociation gives $A + B'$, B being the adaptive enzyme. When a substrate S is present with

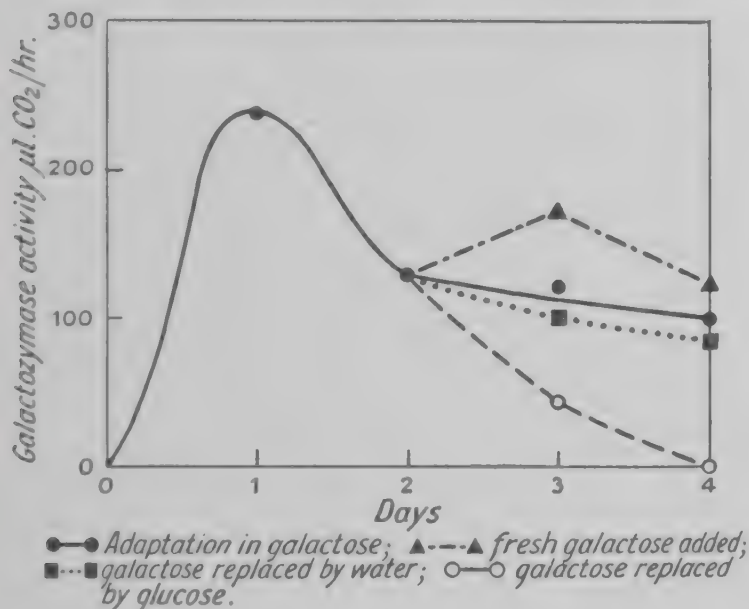


FIG. 6

which B' can combine, the equilibrium $AB \rightleftharpoons A + B'$ is shifted by the new equilibrium $B' + S \rightleftharpoons B'S$, B'S finally dissociating into $B' + S'$, S' being the product of the enzyme action. This process in favourable circumstances continues till B is exhausted, when the maximum production of the adaptive enzyme has taken place.

Such a picture accounts for the facts observed but there are additional ones which need to be considered. In the case of adaptation of yeast to galactose,¹ when the substrate S is removed and becomes exhausted the activity (or amount of) B' (galactozymase) falls off and is restored by addition of fresh substrate (galactose) S (see Figs. 6 and 7) but is brought rapidly to zero by the addition of a new substrate c (glucose).²

It is conceivable that A can control the formation of a group of

¹ Stephenson & Yudkin, 1936.

² Ibid.

enzymes B, C, D, catalysing the decomposition of several related substrates—say different hexoses—the amount of B, C or D being

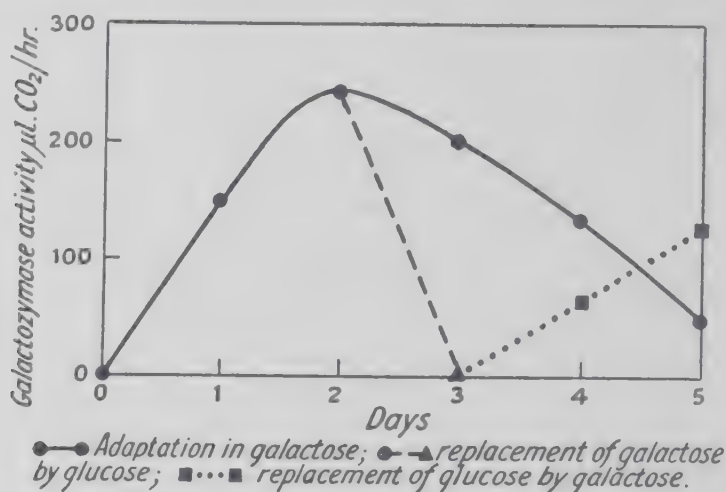


FIG. 7¹

formed by A at any one time being finally controlled by a series of equilibria depending on the substrate present.

The effect of oxygen tension during growth

In general it appears that those enzymes which are active only aerobically are formed preferentially in presence of O_2 , whilst the reverse holds for anaerobic mechanisms. Thus the oxidative deaminases for glycine and alanine are formed preferentially in aerobic conditions; those for serine and aspartic acid, which function best in anaerobic conditions, are formed best anaerobically. The oxidative deaminase for glutamic acid is an exception to this generalisation. The formation of formic hydrogenlyase is strongly inhibited by aerobic conditions and the glucozymase of *Bact. coli* is favoured by anaerobic conditions.

The effect of the pH of the growth medium

It has been shown that *Esch. coli* can grow in media adjusted anywhere between pH 4.5 and 9.0,² but the reaction at which it is grown affects profoundly its enzymic make-up, the enzymes most affected being those concerned with nitrogen metabolism, viz. deaminases and decarboxylases, the latter being strictly adaptive. Broadly speaking, deaminases are formed between pH 7.0 and 8.0 and decarboxylases between pH 6.0 and 4.5. In general the production of decarboxylase is optimal about one pH unit higher

¹ Stephenson & Yudkin, 1936.

² Gale & Epps, 1942.

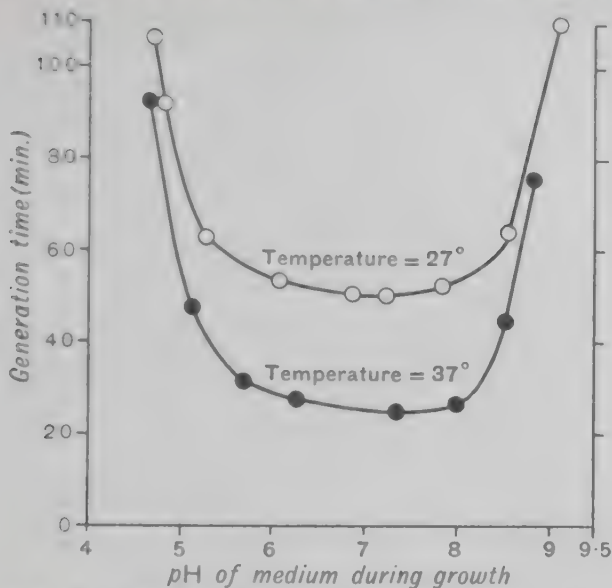


FIG. 8.—Variation of generation time with growth pH (*E. coli*)¹

than the optimal pH for the enzyme, which probably indicates that the pH of the internal environment of the cell is about one unit nearer neutrality than that of the medium.

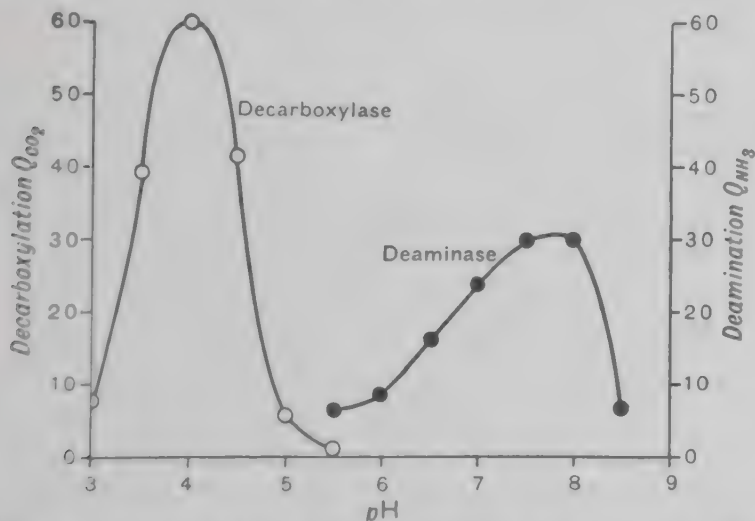


FIG. 9.—Variation with pH of the activities of the enzymes of *Esch. coli* which attack *l*-glutamic acid²

The production of the two enzymes of *Aer. aerogenes* attacking pyruvic acid, viz. the phosphoroclastic enzyme and the carbinol enzyme (see p. 82), is also governed by the pH of the medium,

¹ Gale & Epps, *Biochem. J.*, **36** (1942). ² Gale, *Bact. Rev.*, 1940, **4**, 165.

the former appearing only when the medium is neutral or alkaline the latter when it is acid.

Gale and Epps have studied a number of enzymes in order to show how the optimal pH for the enzyme action is related to the optimal pH for its production. For this study the following definitions were made use of:

pH of optimum activity: that pH at which an enzyme display its highest activity in washed suspension or in a cell-free state.

Potential activity: the activity of an enzyme at its optimal pH .

Effective activity: the activity of a washed suspension or cell free enzyme held at the pH at which the organism is grown.

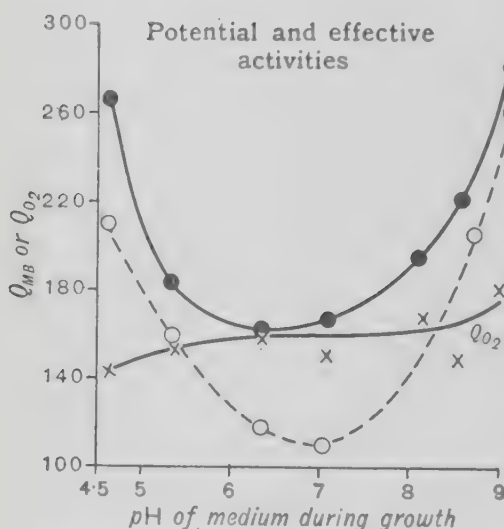


FIG. 10.—Formic dehydrogenase variation of activity with growth pH ¹

●—● Potential activity (Q_{O_2})
 x—x Effective activity (Q_{O_2})
 ○—○ Potential activity (Q_{MB})

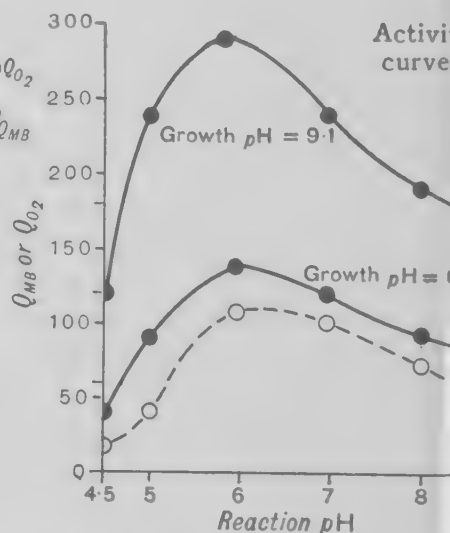


FIG. 11.—Formic dehydrogenase activity pH curves for cultures grown at pH 6.0 and 9.1¹

The enzymes studied fall into two groups, the first comprising those whose effective activity increases as the pH of the growth medium deviates from the pH of optimum activity of the enzyme. Thus during growth the loss in activity due to pH of the medium deviating from the optimal pH of the enzyme is compensated by the production of more enzyme. Six enzymes of this type were studied, viz. formic dehydrogenase, formic hydrogenlyase, alcohol dehydrogenase, catalase, urease and fumarase. With the exception of the last the substrates of these enzymes are all potential poisons. Formate, for example, was shown to inhibit completely

¹ Gale & Epps, *Biochem. J.*, 36 (1942).

the growth of *Esch. coli* at and below pH 6.2. The organism appears to have developed two mechanisms for dealing with this widely spread metabolic product, viz. by formic dehydrogenase and by formic hydrogenlyase. Formic dehydrogenase decomposes formate aerobically and is highly efficient, being one of the most active dehydrogenases known and possessing an extremely high affinity for its substrate. This enzyme is typical of group I, the effective activity being steady between pH 5.0 and 8.0 (see Fig. 10)

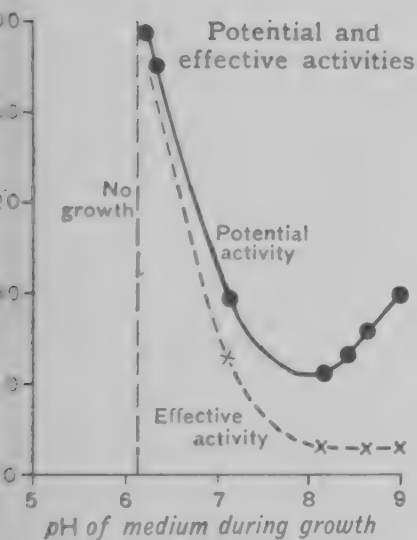


FIG. 12.—Formic hydrogenlyase variation of potential activity $\bullet-\bullet$ and effective activity $x-x$ with growth pH¹.

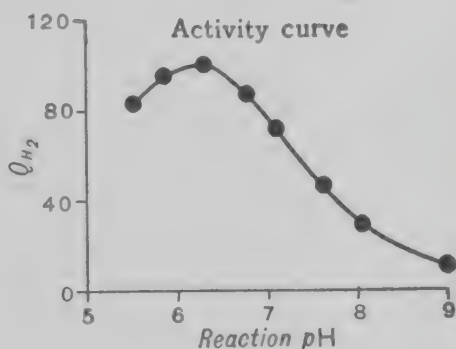


FIG. 13.—Formic hydrogenlyase activity pH curve for culture grown at pH 7¹.

Formic hydrogenlyase, on the other hand, is formed in anaerobic conditions and decomposes formate without the intervention of O_2 or any hydrogen acceptor. Here the potential activity is highest at the pH where the substrate reaches its highest toxicity compatible with the growth of the organism, viz. at pH 6.2.²

In the second group of enzymes there is little or none of the compensating effect found in group I. The enzyme is formed best when the organism is grown at or near the optimum pH of the enzyme. Enzymes falling into this group are hydrogenase, succinic dehydrogenase, glucozymase, tryptophanase, aspartase and the decarboxylases of arginine, ornithine, lysine and histidine and the deaminases of alanine, serine and glutamic acid³ (see Figs. 14 and 15).

¹ Gale & Epps, *Biochem. J.*, 36 (1942).

² *Ibid.*, 1942.

³ *Ibid.*

Temperature during growth

In general, enzyme formation is presumed to occur best at the optimal growth temperature of the organism, but this is not often put to the test. In the case of the amino-acid decarboxylases of *coli* the enzymes are formed more prolifically at 27° than at 37°, this being attributable to their unusual thermolability.

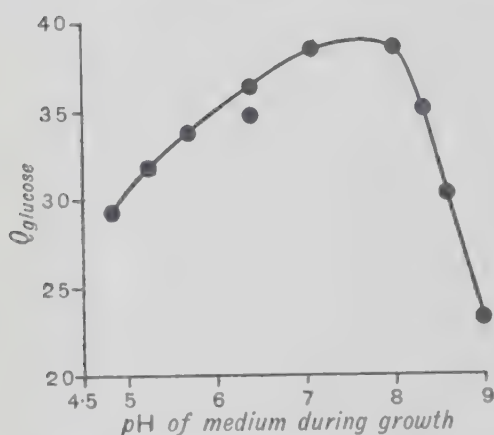


FIG. 14.—Glucosylase. Variation of activity with growth pH
 $Q_{\text{glucose}} = \mu\text{l glucose disappearing/hr./mg. dry weight organism}^1$

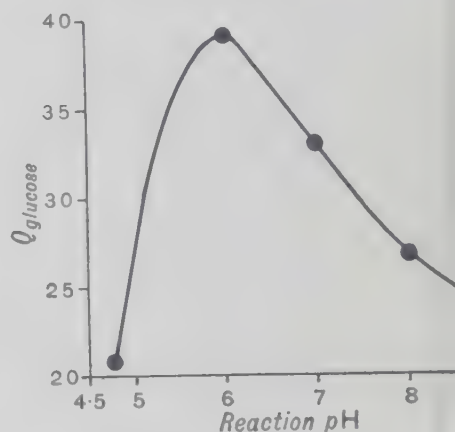


FIG. 15.—Glucosylase. Activity curve for culture grown at pH 7

Effect of carbohydrate in the growth medium

The production of a large number of deaminases is inhibited to the extent of 95% by the inclusion in the growth medium of 2% fermentable carbohydrate. This has been shown in the case of the deaminases of glycine, alanine, glutamic acid, serine, aspartic acid, tryptophan and adenosine triphosphate. The possibility that this effect might be due to anaerobiosis was disproved by direct comparison, but the possibility that it was caused by pH change due to fermentation acids remained longer. This point has now been settled by growing the organism in broth at pH 7.0 and 5.0 and in the presence of 2% glucose, which brought the pH to an average value of 5.2. Table 7 shows beyond doubt that in the case of the deaminases and of ornithine decarboxylase the effect is specifically attributable to the carbohydrate.

¹ Gale & Epps, *Biochem J.*, 36 (1942).

TABLE 7¹

 POTENTIAL ACTIVITIES OF *Esch. coli* WHEN GROWN IN CASEIN DIGEST ADJUSTED TO pH 7.0 OR 5.0 AND CONTAINING 2% GLUCOSE (FINAL pH 5.2 AVERAGE)

Enzyme	Q unit	Potential activity in medium at			Glucose effect
		pH 7.0	pH 5.0	2% glucose	
Hydrogenase . . .	MB	240	136	146	None
Catalase	O ₂	4200	6360	6310	None
Arginine decarboxylase	CO ₂	2	338	272	None
Lysine decarboxylase .	CO ₂	53	194	198	None
Histidine decarboxylase	CO ₂	3	26	33	None
Ornithine decarboxylase	CO ₂	47	560	48	Inhibitory
Alanine deaminase . .	NH ₃	32	4	1.7	Inhibitory
Glutamic acid deaminase	NH ₃	12	3	1.2	Inhibitory
Aspartase	NH ₃	127	247	15	Inhibitory
Serine deaminase . .	NH ₃	855	656	167	Inhibitory
Tryptophanase . . .	Indole	5.4	1.6	0.25	Inhibitory
Alcohol dehydrogenase	MB	52	179	44	Inhibitory
Succinic dehydrogenase	MB	43	23	9	Inhibitory
Formic dehydrogenase .	MB	110	138	58	Inhibitory
Formic hydrogenlyase .	H ₂	75	> 200	139	Inhibitory
Glucosylase	Glucose	38.5	31	77	Stimulatory

Influence of "age" of culture

Much evidence has been accumulated to show that growing cultures show relatively high metabolic activity early in the logarithmic phase. This is based mainly on measurements of O₂ consumed and CO₂ and NH₃ eliminated per viable cell in culture growing in broth medium. Obviously measurements made in growing cultures are affected not only by enzyme variation in the cell but also by continuous change of substrate due to exhaustion of varying constituents of the medium, change in pH, accumulation of metabolic products, partial failure of oxygen supply due to overcrowding, etc. Hence to gain a true picture of the change in enzyme activity of the cell at varying age of culture the cells must be removed, centrifuged and washed and the activities of separate enzymes or enzyme systems determined and related to dry weight or total nitrogen of the cell.

The first reliable data along these lines were supplied by Wooldridge and his co-workers,² who showed that the activity of various dehydrogenase systems of *Bact. coli* varies with the age of culture reckoned from time of inoculation, being low in the early stages, 4-6 hours, and rising to a peak at 20-24 hours. The variability was not constant for all the enzymes studied and was not related to viability. Following this observation many workers

¹ Epps & Gale, 1942.

² Wooldridge *et al.*, 1936; Wooldridge & Glass, 1937.

make it a practice when studying a bacterial enzyme to do a preliminary experiment to determine its variability with age of culture in order that the cells may be reaped in a state of optimal activity. In general it is found that cultures removed at the beginning of the log. phase have low activity which increases linearly and attains a maximum as growth ceases¹ (Figs. 16 and 17). It is not possible

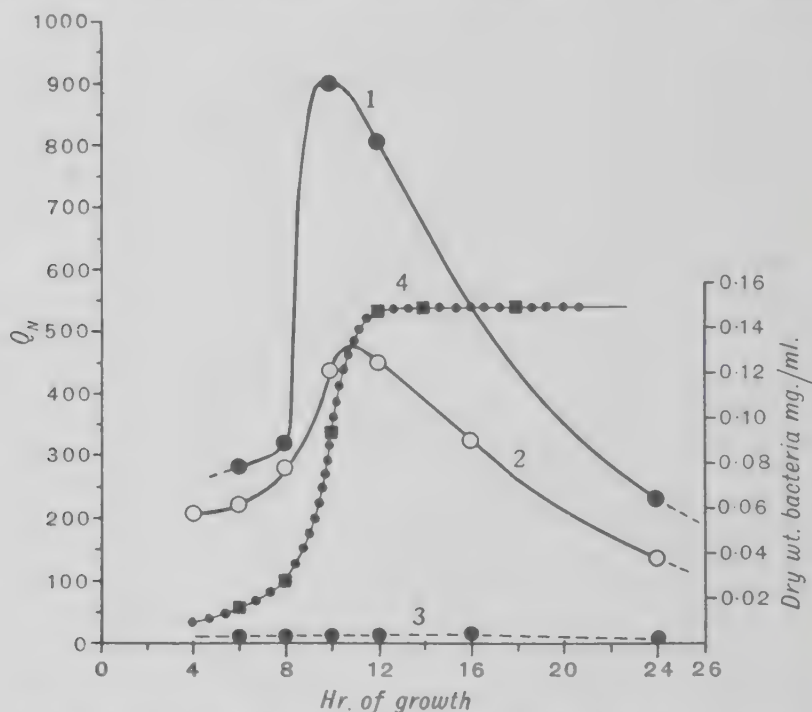


FIG. 16.—Variation of deaminase activity with “age” of culture and conditions of growth; 1 in N_2 , 2 in air, 3 in 2% glucose broth in N_2 , (4) growth curve (strain I)²

to assign any single cause in explanation of these effects. In some cases it is due to the fact that the coenzyme is not present in optimal concentration³ throughout the period; this is the case with the malic dehydrogenase of *Bact. coli*.⁴ Other factors may be removal of toxic substances, exhaustion of growth factors or other chemical changes in the medium. In the case of the aspartase of *Bact. coli* the organism was grown till aspartase activity was well developed. The cells were then removed and the medium resown and aspartase activity determined in the young culture; this proved equal to a normal culture at the end of the growth phase.⁵

¹ Gale, 1943.

³ Ibid., 1938.

² Gale & Stephenson, *Biochem. J.*, **32** (1938).

⁴ Ibid.

⁵ Gale, 1938.

It has been pointed out¹ that the enzymes which have low activity when the organism is dividing most rapidly are those concerned with catabolic reactions. It is conceivable that the enzymes predominating at that period are those catalysing synthetic processes and that there is a turnover to catabolic activity as the cell ceases to divide rapidly.

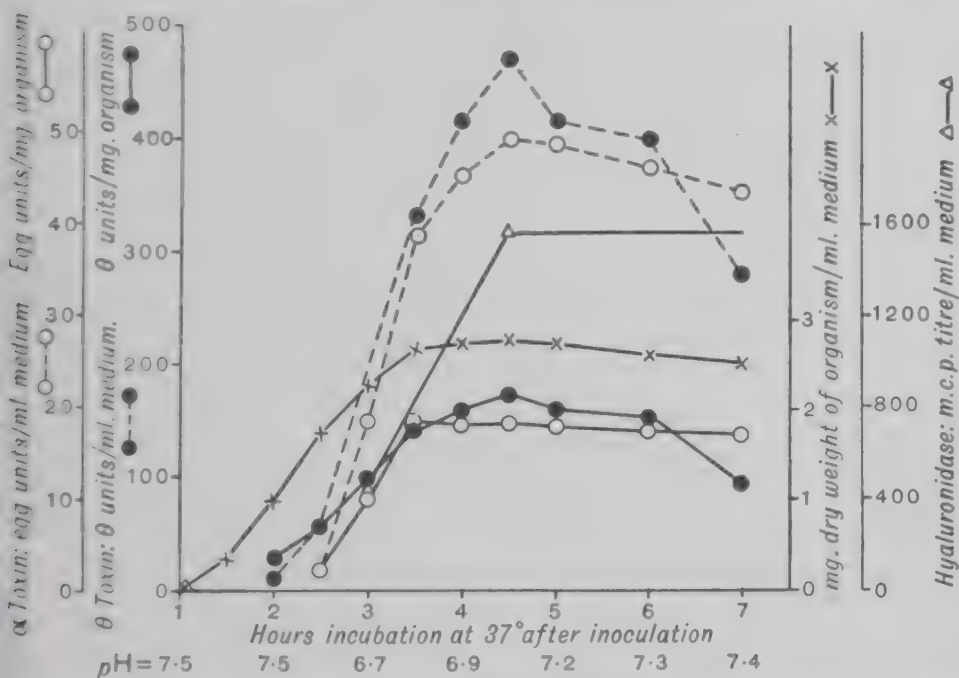


FIG. 17.—Variation with age of culture (*Cl. welchii* S 107) in medium containing 0.45% glucose at an initial pH of 7.5. Growth temp. = 37°⁰²

General considerations

It is impossible to exaggerate the importance of the variability of the bacterial cell or the desirability of studying the laws regulating it. Biochemically, bacterial cells are the most plastic of living material, even as compared with other micro-organisms which have developed a more regulated hereditary mechanism. Higher animals and plants have in addition elaborated machinery for maintaining a constant environment, any severe disturbance in which is reflected in immediate pathological symptoms. The bacterial cell, by reason of its small size and consequent relatively large surface, cannot develop by maintaining a constant chemical environment, but reacts by adapting its enzyme systems so as to survive and grow in changing conditions. It is immensely tolerant of experimental meddling and offers material for the study of pro-

¹ Gale, 1944.

² Gale & van Heyningen, *Biochem. J.*, 36 (1942).

cesses of growth, variation and development of enzymes without parallel in any other biological material. Other living forms have long passed their experimental stage and have evolved relatively satisfactory stable systems ; biochemically speaking the stages of their evolution are unknown to us. But with bacteria constant evolutionary changes occur under our eyes and can be controlled and imitated in the laboratory. Bacterial studies pay the highest dividends on biochemical investigation.

APPENDIX

MEDIA

CHAPTER II

MEDIUM I.¹ (For the isolation of methane bacteria)

NH ₄ Cl	1 g.
K ₂ HPO ₄	0.04 g.
MgCl ₂	0.01 g.
Organic substrate	1-2 g.
Tap water	100 ml.
pH	7.0

CHAPTER III

MEDIUM I.² (*Cellulobacillus myxogenes*)

(NH ₄) ₂ SO ₄	2 g.
K ₂ HPO ₄	1.0 g.
MgSO ₄	0.5 g.
NaCl	0.5 g.
Cellulose	5.0 g.
Water	1000 ml.

The same medium is used for plates with 1-1.5% agar.

*The preparation of cellulose*³

Into a 2-l. flask put 100 ml. conc. H₂SO₄ and 60 ml. H₂O; cool to 60°; add 5 g. filter paper previously moistened, shake till the cellulose dissolves and then dilute quickly to 2 l.; the precipitated cellulose is filtered through a pleated filter and then thoroughly washed first with tap and then with distilled water.

CHAPTER IV

MEDIUM I. (Yeast water)

Extract 100 g. yeast with 1000 ml. boiling water in a steamer for 1 hr. Filter and adjust the pH. If cloudy filter again through Kieselguhr, add any additional source of carbon, e.g. glycerol, and make up to 2000 ml.

MEDIUM II. (Lebedev Juice)

Wash 5 kg. brewer's bottom yeast till the wash water is clear, finish on a Buchner funnel. Dry at 25-30°, using a fan, till it crumbles in

¹ Barker, 1936.

² Simola, 1931.

³ Scales, 1915.

the fingers ; rub it through a wire sieve and finish drying and store. 100 g. dry yeast is incubated with 300 ml. water for 3 hr. at 37° and filtered into a vessel surrounded with ice.

MEDIUM III.¹ (*Cl. acetobutylicum*)

K ₂ HPO ₄	0.05%
KH ₂ PO ₄	0.05%
MgSO ₄ . 7H ₂ O	0.02%
MnSO ₄ . 7H ₂ O	0.001%
Glucose	2%
Asparagine	0.1%
Tryptic digest of casein	0.25%
Tryptic digest of liver	2%
equivalent to 2.5% of fresh liver						

CHAPTER VII

MEDIUM I. (Koser and Rettger)²

NaCl	5.0 g.
MgSO ₄	0.2 g.
CaCl ₂	0.1 g.
KH ₂ PO ₄	1.0 g.
K ₂ HPO ₄	1.0 g.
Glycerol	3.0 g.
Water (ammonia-free)	1000 ml.

Various nitrogenous compounds in the proportion of 1%.

MEDIUM II. (Friedlein)³

NH ₄ Cl	5 g.
Na ₂ SO ₄	5 g.
MgSO ₄	0.1 g.
Potassium phosphate mixture	2.0 g.
Water	1000 ml.

Carbon compounds in varying concentrations. The same medium was used with 1% sodium lactate and varying concentrations NH₄Cl, in this case 1% NaCl was added.

MEDIUM III. (Braun and Cahn-Bronner)⁴

NaCl	5 g.
KH ₂ PO ₄	2 g.
Ammonium lactate	6 g.
Water	1000 ml.
Reaction adjusted with	Na ₂ CO ₃

¹ Davies & Stephenson, 1941.

³ Friedlein, 1928.

² Koser & Rettger, 1919.

⁴ Braun & Cahn-Bronner, 1922.

MEDIUM IV.¹ (*B. typhosus*)

Sodium citrate	4 g.
MgSO ₄ . 7H ₂ O	1 g.
NH ₄ Cl (added separately)	2.5 g.
Glucose (added separately)	25 g.
Water	1000 ml.

Various amino-acids, 0.005%. Cystine, 0.02%. Tryptophan, 0.002%.
(Final concentrations.)

MEDIUM V. ("Ammonia medium")²

KH ₂ PO ₄	4.5 g.
N/1 NaOH	26 ml.
NH ₄ Cl	0.5 g.
(NH ₄) ₂ SO ₄	0.5 g.
Water	600 ml.

The above quantities make 1000 ml. of finished medium; 6 ml. is tubed into each test tube and autoclaved; to each test tube are added the following quantities of sterile solutions:

M/60 MgSO ₄ . 7H ₂ O	0.1 ml.
M/2 Sodium lactate	0.5 ml.
M 2 Glucose (sterilised by filtration)	0.25 ml.
M 2 NaHCO ₃ (sterilised by filtration)	0.5 ml.
Distilled water to final vol. 10 ml.	

The pH of the basal medium after autoclaving is 7.6; the addition of the bicarbonate raises this to 8.0; the final adjustment is effected by the conditions of incubation.

MEDIUM VI. (Wildiers' Yeast Medium)³

Cane sugar	100 g.
MgSO ₄	2.5 g.
KCl	2.5 g.
NH ₄ Cl	2.5 g.
Na ₂ HPO ₄	2.5 g.
CaCO ₃	0.5 g.
Water	1000 ml.

MEDIUM VII.⁴ (General use)

KH ₂ PO ₄	1 g.
MgSO ₄ . 7H ₂ O	0.7 g.
NaCl	1 g.
(NH ₄) ₂ HPO ₄	4 g.
FeSO ₄ . 7H ₂ O	0.03 g.
Water	1000 ml.

pH adjusted to individual requirements.

¹ Fildes, Gladstone & Knight, 1933.

² Gladstone, 1937.

³ Wildiers, 1961.

⁴ Stephenson & Whetham, unpublished.

CHAPTER VIII

MEDIUM I.¹ (*Cl. pastorianum*)

Potassium phosphate	1 g.
MgSO ₄	0.2 g.
NaCl, FeSO ₄ , MnSO ₄	traces
CaCO ₃ (sometimes used)					
Glucose	20 g.
Water	1000 ml.

MEDIUM II.² (*Azotobacter*)

K ₂ HPO ₄	0.5 g.
Mannitol	20 g.
or					
Ca, K or Na propionate	5 g.
Reaction slightly alkaline					
Tap water	1000 ml.

MEDIUM III.³ (*Azotobacter*)

K ₂ HPO ₄	0.5 g.
MgSO ₄	0.5 g.
NaCl	0.5 g.
Fe ₂ (SO ₄) ₃	0.2 g.
Aluminium oxide	0.1 g.
CaCO ₃	0.2 g.
Glucose or mannitol	20 g.
Water	1000 ml.

MEDIUM IV.⁴ (*Azotobacter*)

K ₂ HPO ₄	0.8 g.
KH ₂ PO ₄	0.2 g.
MgSO ₄	0.2 g.
NaCl	0.2 g.
CaSO ₄	0.1 g.
Fe ₂ (SO ₄) ₃	0.01 g.
Glucose	10.0 g.
Water	1000 ml.

MEDIUM V.⁵ (*Nostoc*)

MgSO ₄ · 7H ₂ O	0.2 g.
NaCl	0.2 g.
CaSO ₄ · 2H ₂ O	0.1 g.
FeCl ₃ · 6H ₂ O	0.005 g.
CaCO ₃	0.5 g.
Water	1000 ml.

¹ Winogradsky, 1893.³ Kostytschew *et al.*, 1926.⁵ Allison & Hoover, 1935.² Beijerinck, 1901.⁴ Burk, 1930.

CHAPTER IX

MEDIUM I. (*Nitrosomonas*)

A. Concentrated stock solution of salts

$(\text{NH}_4)_2\text{SO}_4$	20 g.
K_2HPO_4	7.5 g.
KH_2PO_4	2.5 g.
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.1 g.
$\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$	0.1 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.3 g.
CaCl_2	0.2 mg.
Water	100 ml.

Dilute 1/100 in tap water for liquid media and for dialysing plates.

MEDIUM II. (*Nitrobacter*)

Same as A, substituting NaNO_2 for $(\text{NH}_4)_2\text{SO}_4$.

MEDIUM III. (Silica Jelly Plates)

A. As above for Media I and II.

B. HCl 30% v/v.

C. $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ 20%
 or
 Na_2SiO_3 anhydrous 8.5%

D. Acid solution

A I or A II	20 ml.
B	300 ml.
Bromthymol blue	0.04%	5 ml.
Distilled water	300 ml.

Put about 0.2 g. sterile CaCO_3 in each sterile Petri dish. Pipette 20 ml. of C into a beaker and run D in from a burette till neutral (about pH 7.4). Immediately pour the contents of the beaker into a Petri dish. The gel sets in about two minutes. Batches of 25 plates are then put in a deep jar and dialysed against liquid medium kept stirred by a stream of bubbles. The liquid medium is changed frequently until almost free from chloride. The plates may be sterilised by wrapping in a towel and autoclaving at 10 lb. pressure for 15 minutes or the surface may be flamed.¹

¹ For further details see Hanks & Weintraub (1936), *J. Bact.*, 32, 639 and 642.

MEDIUM V.¹ (*Thiobacillus thioparus*)

$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$	5.0 g.
$(\text{NH}_4)_2\text{SO}_4$	0.4 g.
K_2HPO_4	4.0 g.
CaCl_2	0.25 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g.
FeSO_4	0.01 g.
Water	1000 ml.

pH 7.0.

MEDIUM VI.² (*Thiobacillus denitrificans*)

Powdered sulphur	100 g.
KNO_3	0.5 g.
Na_2CO_3	0.2 g.
CaCO_3	2.0 g.
K_2HPO_4	0.2 g.
Ditch water	1000 ml.

MEDIUM VII.³ (*Thiobacillus denitrificans*)

$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$	5 g.
KNO_3	5 g.
NaHCO_3	1 g.
K_2HPO_4	0.2 g.
MgCl_2	0.1 g.
CaCl_2 and FeCl_3	traces
Water	1000 ml.

MEDIUM VIII.⁴ (*Thiobacillus thio-oxidans*)

$(\text{NH}_4)_2\text{SO}_4$	0.2 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1-0.5 g.
FeSO_4	0.01 g.
CaCl_2	0.25 g.
KH_2PO_4	3-5 g.
Powdered sulphur	10 g.
Water	1000 ml.

MEDIUM IX.⁵ (For sulphur organisms of group VI)

$\text{Na}_2\text{S}_2\text{O}_3$	10 g.
K_2HPO_4	4 g.
KH_2PO_4	4 g.
CaCl_2	0.1 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1 g.
$(\text{NH}_4)_2\text{SO}_4$	0.1 g.
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.02 g.
$\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$	0.02 g.
Glucose or asparagine	1-5 g.

¹ Starkey, 1935 (1).² Beijerinck, 1904.³ Lieske, 1912.⁴ Waksman and Starkey, 1912.⁵ Starkey, 1934 (1).

MEDIUM X.¹ (*Leptothrix ochracea*)

Agar	10
Manganese acetate	0.1 g.
Water	1000 ml.

MEDIUM XI.¹ (*Leptothrix ochracea*)

Manganese bicarbonate sat. sol.	100 ml.
NaHCO ₃	0.01 g.
(NH ₄) ₂ SO ₄	0.01 g.
KH ₂ PO ₄ and MgSO ₄	traces
Water	1000 ml.

The manganese bicarbonate is prepared by passing CO₂ into a suspension of manganese carbonate and filtering. Growth is not obtained on a saturated solution ; it is therefore diluted 1 in 10 (as shown above) before use.

MEDIUM XII.² (*Spirophyllum ferrugineum*)

(NH ₄) ₂ SO ₄	1.5 g.
KCl	0.5 g.
MgSO ₄	0.05 g.
K ₂ HPO ₄	0.05 g.
Water	1000 ml.

The medium is sterilised in 100 ml. lots in small Erlenmeyer flasks, the layer of liquid being about 2 cm. deep. After sterilisation the flasks stand at room temperature for two days to absorb atmospheric gases. Iron may be added, previously sterilised, either as iron wire or as iron carbonate ; in both cases the presence of CO₂ results in the formation of ferrous bicarbonate. The organism develops in an atmosphere consisting of ordinary air and 1% CO₂. Incubation at 6°.

MEDIUM XIII.³ (*B. pantotrophus*)

KH ₂ PO ₄	0.5 g.
MgSO ₄	0.2 g.
NH ₄ Cl	1.0 g.
NaHCO ₃	0.5 g.
FeCl ₃	trace
Water	1000 ml.

MEDIUM XIV.⁴ (*B. pycnoticus*)

NaHCO ₃	1.0 g.
NH ₄ Cl	1.0 g.
KH ₂ PO ₄	0.5 g.
MgSO ₄	0.1 g.
NaCl	0.1 g.
Water (glass distilled)	1000 ml.

This medium is sterilised by filtration through a candle.

² Ibid., 1911. ³ Kaserer, 1906. ⁴ Ruhland, 1924.

MEDIUM XV.¹ (*B. oligocarboophilus*)

K ₂ HPO ₄	0.1 g.
NaNO ₃	0.1 g.
MgSO ₄	trace
FeCl ₃	trace
Water	1000 ml.

A supernatant atmosphere saturated with formic acid.

CHAPTER X

MEDIUM I.² (*Thiorhodaceæ*)

NH ₄ Cl	1 g.
K ₂ HPO ₄	0.5 g.
MgCl ₂	0.2 g.
NaHCO ₃	1.0 g.
Na ₂ S . 9H ₂ O	1.0 g.
Water	1000 ml.

pH 8-8.5. Continuous illumination by 50-100 watt lamp at 20-30 cm. Incubation 25-30°.

¹ Lantzs, 1922.

² van Niel, 1931.

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